DIVISION OF DIABETES, ENDOCRINOLOGY, AND METABOLIC DISEASES

FY 1999 Program Plan

RESEARCH PROGRESS REVIEWS

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DIVISION OF DIABETES, ENDOCRINOLOGY AND METABOLIC DISEASES

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Research Progress Reviews

DIABETES MELLITUS

I. TITLE: Intensive Glycemic Control Prevents, Delays or Reverses Complications of Diabetes

BACKGROUND: The Diabetes Control And Complications Trial (DCCT) established that intensive therapy of glycemic control, with a corresponding lowering of HbA1c, dramatically reduced the incidence in type 1 diabetes of microvascular complications, compared with conventional therapy. The DCCT findings showed that intensive therapy aimed at keeping blood glucose levels as close to normal as possible delayed the onset and slowed the progression of diabetic retinopathy, nephropathy, and neuropathy by 35 to 70 percent. The study also conclusively demonstrated that any sustained lowering of blood glucose reduces the risk of developing the microvascular complications of type 1 diabetes. At the close of the DCCT in 1993 all conventional treatment subjects were instructed in the use of intensive therapy and returned to their health care providers. The Epidemiology of Diabetes Interventions and Complications study (EDIC) continues to follow a large group of the original DCCT cohort of subjects to determine the long-term outcome of reduced glycemic exposure.

Although the DCCT was designed to study the effects of intensive therapy in individuals with type 1 diabetes, 90 percent to 95 percent of people with diabetes have type 2 diabetes and its prevalence is increasing. It is projected that worldwide there will be nearly 210 million individuals with this form of diabetes by the year 2010. The UKPDS was designed to determine whether intensive management of type 2 diabetes in controlling blood glucose levels resulted in a reduction in long term diabetes complications compared with standard care.

RECENT FINDINGS: The UKPDS is the largest study of individuals with type 2 diabetes ever performed, lasting 20 years and including 5,102 subjects. The study was designed to test whether intensive blood glucose control in type 2 patients reduced the risk for the macrovascular and microvascular complications of the disease compared to individuals randomized to diet therapy alone. It also tested whether a particular therapy was more effective and what the combined effects of lowering blood pressure and blood glucose were on diabetes and cardiovascular complications. The main clinical findings of this study have been reported in four papers, two in The Lancet and two in the British Medical Journal.

The UKPDS findings demonstrate that intensive control of blood glucose in type 2 diabetes significantly reduces the incidence of both retinopathy and nephropathy by 25 percent compared to standard treatment. Across all treatment groups combined it was also shown that for every 1 percent drop in

HbA1c (e.g. reducing HbA1c from 8.0 to 7.0) there was a 35 percent reduction in retinopathy, neuropathy, and nephropathy. Although there was evidence that intensive blood glucose control reduced the risk for myocardial infarction, the results were not significant for any of the interventions tested.

The results for the blood pressure lowering component of the UKPDS demonstrated the benefits of aggressive control of blood pressure in those randomized subjects that were hypertensive and diabetic compared to the group with less tight control of blood pressure. There was a 32 percent reduction in diabetes related deaths, a 44 percent reduction in strokes, and a 37 percent reduction in diabetes related microvascular end points.

Lachin et al,(1998) and Cleary et al, (1998) report convergence of HbA1c values for the original DCCT intensive and conventional treatment groups from 7.2 percent vs. 9.0 percent respectively during the DCCT to 8.1 percent and 8.3 percent respectively during the EDIC. In spite of this convergence, the former intensive treatment group had a 71-77 percent ($p<0.001$) reduced risk of worse retinopathy after four years of follow up in EDIC. There was also a reduced risk of further progression from the close of the DCCT of 72-87 percent ($p<0.001$), adjusting for the level of retinopathy at DCCT closeout. These measures of retinopathy also included the more advanced stages of severe non-proliferative and proliferative diabetic retinopathy, clinically significant macular edema, and the need for laser surgery for either retinopathy or macular edema. Risk of progression of albuminuria >40 mg/24 hours was reduced 53 percent ($p<0.001$) and progression of albuminuria >300 mg/24 hours was reduced 87 percent ($p<0.001$) in the intensive versus conventional treatment group. The relationship between HbA1c and the risk of further progression of retinopathy was assessed. In the former conventional treatment group, as mean HbA1c during the DCCT increased, there were dramatic increases in the risk of worsening retinopathy; however there was little further effect on the risk of worsening as the EDIC mean HbA1c increased. Among those in the former intensive treatment group, the risk of further worsening was weakly associated with mean HbA1c during the DCCT and not significantly with that during EDIC.

The DCCT research group examined whether there was an effect of intensive versus conventional therapy on residual B-cell function in type 1 patients who when randomized into the DCCT, were determined to have C peptide values that defined them as C peptide responders. At baseline, responders in intensive and conventional treatment groups had similar C-peptide levels. Responders receiving intensive therapy maintained a higher stimulated C-peptide level and had a lower likelihood of becoming nonresponders than responders on conventional therapy. Although C-peptide levels eventually were similar with later years of follow-up, the risk reduction in intensive versus conventionally

treated groups was 57 percent ($p<0.001$) over the mean 6.5- years of follow-up. During the first four years of the DCCT among the intensive treatment group, responders had significantly lower HbA1c levels than nonresponders; this trend continued with longer follow-up but was not statistically significant. Among the intensive treatment group, there was a 50 percent reduction in the risk of retinopathy in responders compared with nonresponders (relative risk=0.50, 95 percent CI=0.28-0.88); after adjustment for HbA1c level, the reduction in risk was no longer significant. Micro-albuminuria also occurred less frequently in intensively treated responders than nonresponders but was not significant. Despite lower HbA1c levels in intensively treated responders, the risk for severe hypoglycemia with seizure or coma was 65 percent (95 percent CI=53-74 percent) less in this group versus intensively treated nonresponders. No differences in the development of complications were seen between the C-peptide responders and nonresponders in the conventional treatment group.

Fioretto et al, (1998) report that in type 1 patients who receive pancreas transplants but have not received kidney transplants, the lesions of diabetic nephropathy were not ameliorated over a 5-year exposure to normoglycemia. However, 10-years after pancreas transplantation, creatinine clearance rate, thickness of the basement membranes, and the mesangial fractional volume decreased significantly to normal levels. The authors conclude that pancreas transplantation can reverse the lesions of diabetic nephropathy, but that this process exceeds 5-years of exposure to normoglycemia and is evident when measured at 10-years.

SIGNIFICANCE: Since the completion of the DCCT, the major findings that a reduction in glycemic exposure reduced the long term complications of type 1 diabetes have been extended beyond the experimental design of the DCCT and to type 2 diabetes. The original cohort of subjects from the DCCT have been followed in the Epidemiology of Diabetes Interventions and Complications (EDIC) study. This surveillance study is being conducted to determine the long term benefit of intensive glycemic management. The findings suggest that intensive therapy aimed at maintaining normal glycemic levels has a beneficial impact on long-term complications that extend beyond the period of intensive therapy. Risk of microvascular complications does not appear to be acutely affected by the prevailing level of hyperglycemia. Rather these risks are associated with the long-term chronic effects of hyperglycemia that take considerable time to become manifest and are equally slow to dissipate with reductions in hyperglycemia. Intensive therapy should be implemented as early as is safely possible in subjects with type 1 diabetes, and maintained as long as possible.

Results from the DCCT cohort of C-peptide responders and non responders

support initiating intensive therapy as early in the course of type 1 diabetes as is practical and safe. Such treatment helps sustain residual endogenous insulin secretion which, in turn, allows better metabolic control for longer periods with fewer acute and chronic complications. The ability to maintain lower HbA1c levels with relatively fewer episodes of severe hypoglycemia makes earlier intervention especially appealing.

The importance of long-term glycemic control is also demonstrated by the reversal of the lesions of diabetic nephropathy following pancreas transplantation into individuals with type 1 diabetes. However, the reversal is not apparent upon the normalization of glycemia measured at 5-years, but is seen 10-years after transplantation.

The importance of glycemic control for type 2 diabetes has been conclusively demonstrated by the UKPDS that tested whether intensive control of blood glucose in type 2 diabetes significantly reduces the risk for developing the microvascular complications of the disease. This landmark study confirms the findings of the NIDDK supported DCCT that any reduction in blood glucose reduces the risk for developing the long-term microvascular complications of type 2 diabetes.

FUTURE DIRECTIONS: The UKPDS will be followed by a 5-year post-study to investigate longer-term responses to the intensive treatment protocol. This will allow the investigators to establish whether the indication of a reduction in risk for myocardial infarction reported in the UKPDS findings will achieve the level of statistical significance.

The Epidemiology of Diabetes Interventions and Complications (EDIC) study is a long-term epidemiologic surveillance study of the original cohort of subjects from the DCCT. At the close of the DCCT, all conventional treatment subjects were instructed in the use of intensive therapy and all subjects returned to their health care providers for further diabetes care. The EDIC study group is continuing to follow EDIC participants for the effects of former treatment group assignment during the DCCT, hyperglycemia, and other risk factors on the development and progression of microvascular and macrovascular disease.

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II. TITLE: Type 1 Diabetes Mellitus: Etiology and Pathophysiology

BACKGROUND: Type 1 diabetes mellitus is an autoimmune disease in which an individual's immune system attacks and destroys its own insulin-producing β-cells in the pancreas. Normally, the immune system functions to protect us

against foreign agents, such as bacteria and viruses. However, in certain circumstances, the immune system identifies a normal part of the body as foreign and initiates a powerful attack to destroy and remove it from the body. Type 1 diabetes results when the pancreatic β-cells are attacked and destroyed.

Type 1 diabetes is a genetic disease with environmental influences. The function of the immune system is determined, in part, by the specific human leukocyte antigen (HLA) genes which each individual inherits. Certain HLA genes have been shown to be associated with a high risk of developing diabetes, while other HLA genes appear to exert a protective effect. The HLA genes influence the function of lymphocytes, which play a major role in the etiology and pathogenesis of type 1 diabetes. There are two predominant types of lymphocytes – β-cells, which produce antibodies, and T cells, which can directly kill cells. Both T and β-cells appear to be involved in the development of type 1 diabetes.

The antibody-producing β-cells gave researchers one of the first indications that type 1 diabetes was an autoimmune disease. Autoantibodies that react specifically against pancreatic β-cells are found in new onset patients with diabetes, as well as in numerous animal models of type 1 diabetes. One of the most well-studied animal models is the non-obese diabetic (NOD) mouse. These autoantibodies are directed against a variety of components of the β-cell. One of these β-cell components, or antigens, is glutamic acid decarboxylase (GAD).

During the activation of naive T cells, a protein on the T cell – the T cell receptor – interacts with a foreign protein or antigen. The set of T cell receptors an individual has is genetically determined. The combination of receptor and antigen appears to determine whether the resulting immune response will be autoimmune in nature. Naive T cells are activated by a multifaceted process requiring the interactions of different sub-populations of T lymphocytes (CD4+ and CD8+), cytokines, foreign antigens (e.g., viral proteins) and the genetic background of the individual. Cytokines are locally synthesized proteins that act on lymphocytes to induce an immune response. During the multiple step activation process, CD4+ lymphocytes can be further subdivided into T helper cell subsets, referred to as Th1 or Th2 cells, depending on which cytokines are secreted. Based on work in the NOD mouse, it has been proposed that the development of type 1 diabetes is controlled by the ratio of Th1 to Th2 cells, with Th1 cells promoting diabetes and Th2 cells protecting against disease.

Environmental factors, such as infections or dietary substances, have been postulated to act as triggers for the autoimmune response. Epidemiological

studies have indicated that Coxsackie B virus infection is very common in individuals who develop type 1 diabetes. Although it has not been definitively proven that Coxsackie B virus can trigger disease, researchers have focused a great deal of attention on possible mechanisms by which the virus could destroy pancreatic β-cells. One hypothesis is that the virus, which directly invades the pancreas, simply causes local inflammation and cell destruction. The inflammed cells, in turn, release autoantigens (e.g., GAD) which are normally sequestered, thus triggering an autoimmune response. This non-specific process has been termed "bystander damage." Another hypothesis, termed "molecular mimicry," is that the autoimmune response is triggered because of sequence similarity between a viral protein and GAD. In essence, the body mounts an attack against the invading virus, but ends up attacking its own β-cells because of sequence homology between GAD and virus.

RECENT FINDINGS: While both theories of how viruses might trigger diabetes are plausible, recent evidence suggests that, in the mouse model, Coxsackie virus appears to cause diabetes by bystander damage and not by molecular mimicry. Investigators infected different strains of mice with Coxsackie B virus, in order to determine if the development of diabetes was dependent on cross-reactivity of the virus with GAD. NOD mice, which develop spontaneous diabetes and show signs of autoimmunity against GAD, did not demonstrate accelerated development of disease when infected with Coxsackie, which would be expected if molecular mimicry was operative. However, a different strain of mice, BDC2.5 (which do not normally develop diabetes), rapidly developed disease after infection with virus. These mice carry T cells that are reactive to a pancreatic antigen distinct from GAD and not cross-reactive with Coxsackie virus. Thus, in these experiments, it appeared that autoimmunity resulted from bystander damage and release of antigens from damaged β-cells, rather than from an attack due to cross-reactive epitopes.

Whatever the trigger for β-cell destruction is, the end result is the appearance of autoantibodies, including those directed against GAD. One important question relates to whether GAD antibodies are merely markers for the autoimmune process, or whether GAD antigen itself is an inciting player in the autoimmune process. In two related studies in the NOD mouse, investigators have shown that CD4+ T cells specifically reactive against GAD can directly produce β-cell injury. Injection of GAD into NOD mice accelerated the development of diabetes. CD4+ T cells isolated from these mice were reactive against GAD and demonstrated an autoimmune Th1 cytokine secretion pattern. In addition, the GAD-specific CD4+ T cells from diabetic NOD mice could adoptively transfer diabetes into mice that would not normally develop the disease. This is the first evidence that GAD may actually have a primary role in the autoimmune process.

Much of the evidence for an environmental trigger of type 1 diabetes comes from epidemiological studies, including those which demonstrate that even identical twins do not have 100 percent concordance for developing disease. Studies of family members of patients with diabetes indicate that many individuals have evidence of autoimmunity (i.e., the presence of β-cell autoantibodies) yet do not develop disease. By studying sets of identical twins and triplets who are discordant for disease, researchers have found that there is an altered pattern of cytokine secretion in those who develop disease. Patients who develop type 1 diabetes secrete decreased levels of the cytokine Interleukin 4, which is known to promote a protective Th2 immune response. This extends previous findings from the NOD mouse to humans and is an important advance in understanding the nature of the autoimmune response.

SIGNIFICANCE: Understanding the mechanism by which the autoimmune process is triggered is essential for designing prevention/intervention strategies. For example, an approach focused on cross-reactive viral epitopes would be inappropriate if molecular mimicry is not operative.

The demonstration that the immune response against GAD appears to play a primary role in the development of diabetes, rather than merely reflecting pancreatic cell damage, offers a potential target for immunomodulation to prevent or ameliorate disease. Indeed, studies in the NOD mouse (see Type 1 Diabetes: Immunomodulation for Prevention and Therapy) suggest that GAD administration might be an effective therapeutic regimen.

Type 1 diabetes occurs because of a complex interplay between genetics, T cell activation and environment. Dissecting the role of different cytokines in the development of disease is critical to being able to manipulate the immune process away from a destructive, autoimmune pathway. In addition, differential cytokine secretion may provide a marker, which could predict which individuals with antibody evidence of ongoing autoimmunity are most likely to progress to actual disease. Such a marker would be useful for identifying those individuals who might best benefit from early intervention strategies to prevent the development of disease.

FUTURE DIRECTIONS: Our knowledge of the etiology and pathophysiology of type 1 diabetes has been expanded by these recent investigations. Much more work is needed to define the role of viruses and other environmental factors that may trigger the autoimmune process. Identification of such a trigger (e.g., a clear-cut viral etiology) could lead to an effective intervention (e.g., vaccination) to prevent the development of type 1 diabetes. Future research must also be aimed at refining our understanding of the role of cytokines and coactivators in controlling T cell subsets. The long-term goal would be to develop the ability to

control or alter the T cell phenotype, to intervene in the disease process. Only by understanding the pathophysiology of the autoimmune process in the development of type 1 diabetes, will we be able to develop modalities to prevent or treat the disease.

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III. TITLE: Type 1 Diabetes Mellitus: Immunomodulation for Prevention and Therapy

BACKGROUND: Type 1 diabetes mellitus is an autoimmune disease that

affects approximately 800,000 people in the United States. It is one of the most frequent chronic diseases of children with about 30,000 new cases diagnosed each year.

Prevention of type 1 diabetes is a major goal of research. In the past few years, investigators have been able to establish screening tests to identify individuals at high risk for the development of type 1 diabetes. They have also shown in animal models and in preliminary human trials, that low-dose insulin therapy may prevent or delay the onset of the clinical manifestations of type 1 diabetes. A controlled multi-center clinical trial is underway to assess the efficacy of parenteral or oral insulin to prevent or delay the onset of type 1 diabetes.

The search for therapies to prevent the autoimmune attack that eventually leads to type 1 diabetes has indicated the need for therapies that are highly specific for the disease process and for the individual. Current therapies would require an individual to be on systemic immunosuppression for their entire life. One of the screening tests presently utilized to identify individuals at high risk for the disease is genetic and defines the specific human leucocyte antigen (HLA) of the individual. Particular HLA genes [in animal studies these genes are referred to as the major histocompatibility complex, MHC] have been shown to have a high predictive value for identifying the development of an autoimmune attack on the β-cell. By combining specific varieties (alleles) of the HLA genes into an animal model [i.e., the non-obese diabetic (NOD) mouse], researchers can then study preventive therapies designed for the genetic background of the individual at high genetic risk for type 1 diabetes.

Lymphocytes play major roles in the cause and progression of type 1 diabetes. There are two predominant types of lymphocytes: β-cells, which produce antibodies, and T cells, which can directly kill a cell. Both cell types appear to be involved in type 1 diabetes. The antibody-producing β-cells of the immune system gave researchers one of the first indications that type 1 diabetes was an autoimmune disease. The presence of antibodies that react specifically against β-cells was found in new onset individuals with type 1 diabetes and in animal models of type 1 diabetes (i. e., the NOD mouse and the BB/Wor diabetic rat). The proteins or antigens with which these antibodies interact may signify important participants in the disease process. One of these antigens has been identified as glutamic acid decarboxylase (GAD). Naive T lymphocytes are activated by a multifaceted process requiring the interactions of different sub-populations of T lymphocytes ($CD8^+$ and $CD4^+$), cytokines, foreign antigens (viral proteins) and the genetic background of the individual. Cytokines are locally synthesized proteins that act on lymphocytes to induce an immune response. During the multiple step activation process, $CD4^+$ lymphocytes can be sub-divided further into T helper cell subsets referred to as Th1 cells and Th2 cells.

It has been proposed that the development of type 1 diabetes is controlled by the ratio of Th1 to Th2 cells with the Th1 cells promoting diabetes and the Th2 cells protecting against the disease. Thus, researchers are interested in modulating the immune system enabling them to convert a Th1 destructive response to a benign Th2 response.

RECENT FINDINGS: The role of the major histocompatibility complex (MHC) in the NOD mouse model was examined by mutating the two amino acids in the MHC gene which are believed to cause this animal model to be susceptible to type 1 diabetes. The transgenic mice did not develop diabetes even after 8 months. Thus, this MHC gene plays a role of altering the immune regulatory networks.

Intravenous administration of GAD65 to 12-week old NOD mice suppresses the autoimmune attack on the β-cell. NOD mice at this age have an ongoing insulitis, which progresses to overt type 1 diabetes between 13 and 25 weeks of age. The GAD65 treatment effectively prevents this disease progression. This prevention of diabetes is mediated by the induction of regulatory $CD4^+$ T cells, which have a Th2 phenotype.

Type 1 diabetes is believed to be initiated by the development of autoreactive T cells reacting to a specific portion or determinant of an antigen, for example GAD65. With time the immune response spreads to additional determinants. This process is referred to as determinant spreading. Using an assay capable of characterizing T cells at the single cell level, the natural development of β-cell autoimmunity and the development of tolerization to a β-cell antigen in NOD mice was examined. These observations confirmed determinant spreading of a Th1 response during the spontaneous autoimmune process. They also demonstrated a new phenomenon, Th2 determinant spreading, which may be a mechanism underlying the efficacy of antigen-based immunotherapies.

Using a viral-induced murine model (lymphocytic choriomeningitis virus) of type 1 diabetes, researchers have shown disease prevention by developing transgenic mice with the immunoregulatory and cytokine inhibitory genes (E3) of the adenovirus genome. These results predict that the selective immune regulation at the level of the target cell is sufficient to prevent autoimmune diabetes without disrupting the function of the systemic immune response.

SIGNIFICANCE: Predisposition of an individual to type 1 diabetes is controlled by his HLA genes. Understanding how this control is manifested will empower researchers to intervene with specific prevention protocols.

Once the autoimmune attack on the β-cell has begun, intervention may be more

difficult. Methods to monitor the individual's response to a therapy are essential. These results indicate a method to induce tolerance in an animal model, thus preventing further autoimmune attack, and a procedure to monitor the effectiveness of the intervention by examining the autoimmune reactivity of single T cell responses in the periphery.

Selective immune regulation at the level of the β-cell is significant for several reasons. First, these results suggest if vectors are designed to transfect only the surviving β-cells, this strategy could give these cells the machinery necessary to inhibit the ongoing autoimmune attack. Second, β-cells could be transfected with the genes that offer immune protection *in vitro*. After the transfection, these cells could be utilized for transplantation into an individual with type 1 diabetes without fear that a continuing autoimmune process in the individual would destroy them.

FUTURE DIRECTIONS: Immunomodulation for the prevention and treatment of type 1 diabetes has made considerable advances in animal models. However, it is essential to move these findings forward into the clinical situation. Development of protocols to intervene in the disease process in humans and development of assays to monitor responsiveness to therapies in humans are essential. Validation of a surrogate endpoint in humans would decrease the time required to move a given prevention/intervention protocol forward. Research in these directions must continue.

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IV. TITLE: Type 1 Diabetes Mellitus: Mechanical and Cellular Approaches to Therapy

BACKGROUND: Type 1 diabetes mellitus is an autoimmune disease that affects approximately 800,000 people in the United States. It is one of the most frequent chronic diseases of children with about 30,000 new cases diagnosed each year.

Currently available intensive insulin therapy is limited by the accompanying increased frequency of severe hypoglycemia and weight gain, resulting in an increasing prevalence of overweight patients with type 1 diabetes. Intensive therapy is also particularly problematic in children and adolescents due to difficulties in getting them to comply with current treatment regimens. This is especially troublesome since current clinical data support early intervention as being the most effective treatment strategy. Because, the results of the Diabetes Control and Complications Trial indicate that maintenance of near normal glycemic levels can reduce and delay the onset of the devastating complications of diabetes, establishing methods to achieve and maintain euglycemia will have enormous impact on the health and quality of life of individuals with diabetes.

Although the ultimate research goal is to prevent the onset of type 1 diabetes, investigators are currently developing approaches and technology to modulate blood glucose levels in patients with diabetes and to restore insulin-producing capacity through transplantation of the whole pancreas, or of islets from the pancreas. Today, the only method that offers normal blood glucose levels is pancreas transplantation. Several decades ago, islet transplantation was proposed in the hope of reducing the need for difficult surgical procedures by

allowing intravenous injection of islets. Initial animal studies on islet transplantation were encouraging. Unfortunately, the initial promise held out for islet cell transplantation has not been realized. Thus, of the 270 adult islet transplants performed by the end of 1995 in patients with type 1 diabetes, only ten percent of recipients did not require insulin injections for more than one week, and only five percent remained off exogenous insulin for more than one year. In light of these results, studies are needed to better understand what treatment regimens would allow successful islet transplantation using the least amount of immunosuppression, thereby minimizing the toxicity to the islet. For example, corticosteroids, a mainstay of immunosuppressive regimens for solid organ transplantation, interfere with the normal β-cell function to release insulin in response to elevated blood sugar levels. In addition, the recurrence of autoimmune- mediated destruction of transplanted β-cells is problematic, and many researchers are pursuing studies to abrogate the underlying autoimmunity that has led to type 1 diabetes.

While the "gold standard" for transplant success has been insulin independence, clinicians are observing a beneficial effect of islet transplantation even in recipients who have not achieved insulin independence. These patients require less exogenous insulin and show an improvement in metabolic control, which equates to fewer episodes of severe hypoglycemia. This effect may also lower the risk of long-term complications.

Other β-cell replacement strategies include bioengineered β-cells, β-cells grown in continuous or permanent culture to expand the number available for transplantation, and animal islets (xenotransplants). Bioengineered β-cells could be non-beta cells that would be transfected with specific genes to mimic β-cell function, could be "real" β-cells engineered to enhance engraftment or prevent rejection and autoimmune destruction, or could be a combination cell which functions to release insulin in response to glucose and prevents graft rejection. Animal islets, while offering an ample supply of islets, present additional risks, such as the transmission of animal diseases to humans and accelerated immune rejection.

Glucose sensors for the treatment of diabetes hold great promise for improving metabolic control and quality of life for persons with diabetes. The single greatest change in the management of both type 1 and type 2 diabetes in the past two decades has been the introduction and widespread implementation of reliable, accurate, and relatively "user-friendly" self-glucose monitoring devices. At present, state-of-the-art technology cleanly divides mechanical delivery devices and glucose sensing technology; however, the ultimate goal would be to develop a "closed-loop" delivery system by combining these two technologies.

Despite the enormous success of self-glucose monitoring, the technical challenges of developing methods for continuous monitoring of blood glucose and several highly publicized industry failures have overshadowed recent progress in the field of glucose sensing. Several approaches for continuous glucose measurement are close to clinical applicability. Designs that utilize an enzyme electrode (such as glucose oxidase) and either a hydrogen peroxide or an oxygen detection system appear to be the most successful to date. These electrodes can be placed subcutaneously or intravenously. Other glucose sensor designs being examined include acute microdialysis systems, transdermal extraction of tissue fluids for glucose assay, and non-invasive technologies. These designs require more basic research before demonstration of feasibility and subsequent development.

RECENT FINDINGS: Pancreas transplantation is usually considered for individuals with diabetes who require a kidney transplant. However, successful pancreas transplantation in individuals with diabetes without overt kidney disease can reverse the lesions of diabetic nephropathy. This reversal requires between five and ten years of normal glucose levels. This is in spite of the nephrotoxic effects of current immunosuppressive agents. Thus, the devastating effects of diabetes may be reversible if improved immunomodu-lation methods can be applied to pancreas or islet transplantation.

A long-term follow up (six years) of islet allografts in individuals with type 1 diabetes showed improvement in metabolic control even if an individual was not completely insulin independent. These immunosuppressed individuals had improved HbA_{1C} without the severe hypoglycemic episodes observed in the Diabetes Control and Complications Trial (DCCT). Consequently, even a partially successful islet transplant may offer a significant decrease in the progression of diabetic complications.

Human fetal islet-like clusters (ICC) transplanted into the kidney or the pancreas of a nude mouse (animal lacking T cells) were able to grow and mature producing sufficient insulin to restore euglycemia using 15,000 ICCs/kg. Transplantation into the lung, the liver or the spleen did not result in substantial growth or differentiation of the clusters. Thus, the site for transplantation of human fetal islet-like clusters is important, with advantages to transplantation either under the kidney capsule or in the pancreas.

Induction of immune tolerance has been achieved in the xenotransplantation of rat islets into mice treated with donor-specific spleen cell transfusion and anti-CD154 monoclonal antibody. Since the anti-CD154 antibody is directed against the CD40 ligand which is part of the co-stimulatory signal expressed by activated $CD4^+$ T cells, these T cells must play a major role in the cellular immune

response to xenografts. Further studies suggest that a skin xenograft (considered more difficult to maintain without rejection) can survive on adult thymectomized mice treated with a donor-specific transfusion and a short-term

course of anti-CD154 monoclonal antibody. Thus, it appears that a durable allotolerance can be achieved without prolonged immunosuppression.

Technological advances have occurred in the area of glucose sensors. Glucose sensors coated with two cross-linked polyethylene glycol derivatives (hydrogel) were shown to have improved biocompatibility. Unlike prior coatings that can become encapsulated by tissue *in vivo*, the hydrogel coatings have very few adherent cells. Glucose sensors can be calibrated using as few as one calibration point. An algorithm for predicting blood glucose concentration from the subcutaneous glucose concentration, measured by subcutaneous-implanted glucose sensors, has been shown to improve blood glucose estimations.

A new chemical sensor for glucose is being developed. This sensor is a crystalline colloidal array of polymer spheres polymerized within a hydrogel that swells and shrinks reversibly in the presence of glucose. The material changes color in response to glucose. The hydrogel contains a glucose-recognition group. As glucose binds to this group the hydrogel expands causing an increase in the spacing between the crystalline colloidal array spheres. This causes a shift in the diffracted light to longer wavelengths. Future development of this technology will be necessary to apply it to the clinical situation.

SIGNIFICANCE: The DCCT demonstrated the importance of glucose control to prevent the complications of diabetes. Now we know from the pancreas transplant study that long-term maintenance of normal blood glucose control can reverse kidney damage caused by diabetes of long duration. Islet transplantation has been investigated as a means of normalizing blood glucose levels. However, the results to date, based on achievement of insuln independence have appeared disappointing. The present results indicate that even if an islet transplant recipient is not insulin independent, they may receive considerable benefit from the transplanted islets.

Eventually if islet transplantation is successful, sources, other than human cadaver, will have to be utilized to treat all individuals with diabetes. The ability to transplant human fetal islet-like clusters and have them grow and differentiate into islets *in situ* demonstrates one possible source for additional islet tissue. Several investigators have suggested the use of porcine islets as an additional source. For this to be successful, it is essential to develop immune therapies to circumvent both the autoimmune process and the xenograft rejection process. The present studies examine protocols that are effective for rat to mouse

transplants. Testing these protocols in larger animals will be crucial prior to their evaluation in humans.

The development of a glucose sensor has been a very challenging endeavor. This field is moving rapidly forward. A least one company has submitted a subcutaneous glucose sensor to the Food and Drug Administration for approval. Several other subcutaneous sensors are approaching this evaluation. All of the subcutaneous glucose sensors will require an algorithm to convert subcutaneous glucose levels to blood glucose levels. Such an algorithm is now available for testing. In addition, innovative ideas are being applied to develop noninvasive sensors.

FUTURE DIRECTIONS: The future of transplantation in the treatment of diabetes is extremely exciting. New methods to induce immune tolerance have been developed and are being tested in humans using islets isolated from cadaver pancreata. However, it is clear that additional sources of islets will be essential to treat all individuals with diabetes. Thus, it is essential to develop new sources of islets from human fetal tissue, other animals, or bioengeneered cells in culture. Equally important will be the testing of new tolerance induction protocols to utilize these new islet sources without the need for long term immunosuppression.

The progress toward the development of a glucose sensor must be further advanced. We are only beginning to attract the expertise necessary to initiate novel devices that will be the next generation of glucose sensors. In addition, it will be necessary to evaluate the present glucose sensors to ascertain their usefulness to monitor blood glucose levels and to predict hypoglycemia. Once a sensor has passed these tests, it should be linked to glucose delivery system and further tested. Clearly substantial work is required to produce a closed-loop system. However, the recent progress in this area supports our enthusiasm for continuing research efforts designed to produce a closed-loop system.

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V. TITLE: Type 2 Diabetes Mellitus: Etiology and Pathophysiology

BACKGROUND: Type 2 diabetes mellitus affects more than 16 million Americans and is the predominant form of diabetes in the United States, with a disproportionate impact upon minority populations. It is a multifactorial disease with a significant genetic component, although the specific genes that underlie most cases of type 2 diabetes have remained elusive. In some families with a particularly rare form of type 2 diabetes, Maturity-Onset Diabetes of the Young (MODY), the gene for glucokinase, a key enzyme of glucose homeostasis found in the insulin-secreting β-cell of the pancreas and in the liver, has been implicated. Mutations in glucokinase, along with perhaps a half-dozen other mutations (e.g., in insulin, the insulin receptor, glycogen synthase, insulin-stimulated glucose transporters, other candidate genes, or in the mitochondrial genome), may account for a few percent of type 2 diabetes cases. Since the genetics of type 2 diabetes in humans follows a complex pattern of inheritance, it has been theorized that type 2 diabetes is not caused by a single mutation in one major gene. Thus, type 2 diabetes may develop as a result of different mutations at different genetic loci acting simultaneously and in a synergistic manner (epistasis). For the most part, however, information is still urgently needed about the interplay between genetic and environmental factors in producing full-blown type 2 diabetes, as well as its precursor, insulin resistance.

Type 2 diabetes is characterized by a complex and variable phenotype and often a long period of latency between the appearance of the first recognizable marker(s) and full expression of the disease. There is no doubt that type 2 diabetes has two basic components, peripheral insulin resistance and defective insulin secretion. The insulin resistance is postulated to result from alterations in the insulin signaling pathways, which allow the target cell to respond to insulin and increase glucose uptake. Defective insulin secretion results from a decrease in the signaling mechanisms of the β-cell that allows it to respond to elevated glucose levels and secrete insulin. Breakdown in both of these cellular response pathways occurs in type 2 diabetes and ideally correction of both abnormalities must occur to effectively treat this disease. The clinical course of this disease, as observed in the natural history studies on Pima Indians,

includes a preclinical stage in which the body increases its production of insulin in order to maintain blood glucose levels within the normal range while in an insulin-resistant state. As the disease progresses, insulin levels begin to fall and the patient becomes frankly diabetic. While significant

progress has been made in understanding the biochemical basis for, and genetic influences on, the development of type 2 diabetes, there also appear to be environmental and life-style factors that place individuals at higher risk for type 2 diabetes.

In mammals, insulin is the principal hormone controlling blood glucose and acts by stimulating glucose uptake and metabolism in muscle and fat tissue and inhibiting glucose production in the liver. Insulin action is mediated through the insulin receptor, a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity. The mechanism causing insulin resistance is poorly understood. It is however a major feature of type 2 diabetes and results in a failure of target tissues to respond to normal levels of circulating insulin. Hence, to understand control of normal glucose metabolism, as well as the pathogenesis of type 2 diabetes, it is critical to understand the signaling pathways used by the insulin receptor and how abnormalities in this produce insulin resistance. It is now clear that addition or subtraction of a phosphate moiety to a protein or an enzyme plays a major role in the cellular response to insulin. Thus, enzymes that add phosphate moieties (kinases) and enzymes that remove these moieties (phosphatases) are integral constituents of insulin response pathways. The insulin receptor has kinase activity and can phosphorylate itself upon binding insulin. The insulin receptor also phosphorylates a series of proteins, including insulin receptor substrate-1 (IRS-1) and IRS-2. Once phosphorylated, IRS-1 has been shown to stimulate the phosphatidylinositol (PI) 3'-kinase pathway in liver and skeletal muscle, initiating a cascade of events leading to the cellular response. A primary effect of insulin signaling in muscle is the increase of glucose uptake. Insulin resistance leading to type 2 diabetes may be mediated by deficiencies in glucose transport. GLUT4 is the major insulin-responsive glucose transporter in skeletal muscle, heart and adipose tissue. In contrast, another glucose transporter, GLUT1, is found constitutively on the cell surface of the same tissues, and is not responsive to insulin regulation. Insulin regulates GLUT4 activity in two ways. Short-term regulation occurs via recruitment of GLUT4 from its storage site in intracellular vesicles to the plasma membrane. Long-term exposure to insulin results in downregulation of GLUT4 transcription. Defects anywhere along this signaling pathway from receptor phosphorylation to GLUT4 translocation may lead to increased insulin resistance.

Recent data have implicated tumor necrosis factor-α (TNF-α) in the insulin resistance of obesity and type 2 diabetes, and have suggested that TNF-α

participates in obesity-related systemic insulin resistance by inhibiting insulin receptor tyrosine kinase activity. In obese humans, adipose tissue TNF-α levels are increased compared to controls and are correlated with hyperinsulinemia. Both TNF-α and insulin levels decline with weight reduction, suggesting a role for the abnormal regulation of this cytokine in the pathogenesis of obesity-related insulin resistance. In mice where TNF-α
and its receptor have been knocked out, obesity no longer induces insulin resistance, lending further credence to this hypothesis. An additional component involved in the linkage of obesity to insulin resistance has been identified. This component is the adipocyte fatty acid binding protein, *a*P2. This protein binds free fatty acids and may be involved in the trafficking of fatty acids to specific cellular compartments where the fatty acids elicit their effects on gene expression. Thus, *a*P2 is seen as a co-factor that enables elevated free fatty acids to increase the expression of TNF-α which in turn interferes with insulin action. These studies are beginning to link the metabolic abnormalities associated with obesity to the progression from insulin resistance to diabetes. In particular, it is now appreciated that triglycerides and fatty acids play a major role in this disease. These compounds, generally considered to be energy storage molecules, also control metabolic pathways utilized to metabolize glucose. The recently obtained knowledge of the role of mitochondrial uncoupling proteins in energy dissipation versus energy storage has added another dimension to our appreciation of the metabolic complexities involved in obesity, insulin resistance and diabetes.

Glucose-stimulated insulin secretion from the pancreatic β-cell is also diminished or lost in type 2 diabetes. However, unlike type 1 diabetes mellitus, the β-cells generally remain intact and, in fact, the β-cell mass appears to significantly increase in states of insulin resistance and type 2 diabetes. Significant progress has occurred in the past year in elucidating the role of triglycerides and fatty acids in the loss of β-cell function. In the presence of elevated intracellular fatty acid levels the β-cell loses its ability to respond to glucose. Leptin, an adipocyte hormone important for the regulation of body composition, has been recently shown to have direct effects on β-cells. Leptin increases the metabolism of intracellular fatty acids to energy dissipation rather then to energy storage in the form of triglycerides. Thus, leptin appears to have a beneficial effect on the β-cell in obese/insulin resistant states. Future studies will determine if this hormone may be an effective therapy for improving β-cell function in type 2 diabetes.

Recent findings shed light on the molecular mechanisms for both these phenomena.

RECENT FINDINGS: A number of investigators have utilized the power of

molecular ablation to study various components of the insulin signaling pathway that may play a role in type 2 diabetes. Last year, a group at the Joslin Diabetes Center in Boston, working with NIH intramural scientists, demonstrated in a landmark paper that diminished function at multiple steps in the insulin signaling pathway could lead to the development of diabetes. This provided a plausible multistep model in the development of diabetes in humans. Recently, they demonstrated that elimination of just one of the signal coupling molecules, IRS-2, can produce type 2 diabetes. Mice that have no IRS-2, but express normal levels of IRS-1 are characterized by insulin resistance and a progressive loss of pancreatic β-cell function leading to diabetes. In contrast, animals lacking IRS-1 are able to compensate for peripheral insulin resistance with β-cell hypertrophy and increased insulin secretion to maintain relatively normal glycemia.

Conversion from insulin resistance to frank diabetes occurs with the failure of the pancreatic β-cell to compensate with increased output of insulin. To understand the mechanism which results in the reduction of insulin secretion and eventual loss of β-cell mass, Roger Unger's group in Dallas examined the role of increased free fatty acids in obese Zucker rats. By studying islets from prediabetic and diabetic Zucker rats in vitro, they were able to demonstrate a progressive buildup of free fatty acids within the islets which eventually overwhelms the capacity to oxidize it. The high concentration of fat leads to increased nitric oxide levels and concomitant induction of β-cell apoptosis, or programmed cell death. Why don't all obese individuals who have marked insulin resistance go on to lose their β-cells and develop diabetes? It remains to be demonstrated that this mechanism acts in humans, and whether some individuals are resistant to fatty-acid induced apoptosis.

Because fatty acid metabolism is disturbed in type 2 diabetes, regulation of the oxidation pathway is an area of active research. One of the rate-limiting enzymes is carnitine palmitoyl-transferase I (CPT I), which carries long-chain fatty acids into the mitochondria so they can be used to produce energy. Kelly and colleagues recently showed that the amount of CPT I in normal heart is regulated by its substrate, long-chain fatty acids, but not by other, short-chain fatty acids. The putative fatty acid responsive element was shown to bind to a transcription factor called PPARα. Mice missing PPARα had only 50 percent the control levels of CPT I expression. It is possible that this pathway is impaired in diabetes.

Islet duodenum homeobox-1 (IDX-1) is a transcription factor whose elevated expression correlates with elevated insulin levels, while the CCAAT/enhancer binding protein β (C/EBP-β) factor inhibits insulin gene transcription. IDX-1 and insulin are reduced and C/EBP-β is increased in two hyperglycemic animal models of diabetes, Zucker diabetic fatty (fa/fa) rats, and after 90 percent

pancreatectomy. These findings are consistent with the notion that these transcription factors may mediate long-term down-regulation of the genes that encode for insulin in diabetes. Further support was provided by Dutta and others who also examined the relationship between IDX-1 (also known as PDX-1) and insulin levels. They reduced PDX-1 levels by making transgenic mice with only one copy of the PDX-1 gene. In these animals, insulin levels are inappropriately low for the level of glycemia. In addition, the islets in these animals contain a disproportionately high percentage of non-β cells. Conceivably, gene expression patterns could be reset through appropriate modulation of transcription factor activity. To that end, the pharmaceutical industry has recently embraced transcription factors as potential targets for drugs.

The low number of available animal models of type 2 diabetes has limited the ability to study insulin resistance. A potentially useful model is the heterozygous GLUT4 knockout mouse (GLUT4+/-) which has a reduced number of the insulin-responsive glucose transporter protein, GLUT4. As it ages, this animal develops increased postprandial plasma glucose and insulin, reduced muscle glucose uptake, hypertension, diabetic hypertrophic cardiomyopathy and liver steatosis, without obesity. This indicates that a single impairment in insulin-stimulated glucose transport can initiate a process that leads to diabetes.

A focus on mutated GLUT4 as a locus for type 2 diabetes has yielded data that imply the existence of new factors that regulate GLUT4 transcription and insulin-stimulated movement in the cell. Lee and Jung investigated the role of the cytoplasmic domain of the GLUT4 protein in its movement between storage vesicles and its active location in the plasma membrane. Small proteins corresponding to the cytoplasmic domain were placed into fat cells at a very high concentration. Interestingly, these small fragments caused a dose-dependent increase in recruitment of native GLUT4 to the plasma membrane, similar to that caused by insulin. This implies that there is a regulatory interaction between an unknown molecule in the cell and the cytoplasmic domain of GLUT4 which tags it for cycling back to its intracellular storage site. When there is enough of this protein fragment present to completely bind the putative regulatory factor, GLUT4 remains in its active form at the cell surface. This factor remains to be isolated and characterized. Cooke and Lane found that the suppression of GLUT4 transcription by insulin is likely due to novel nuclear proteins. They transfected adipocytes with various lengths of the GLUT4 gene, all of which contained a special reporter gene. The region of the gene between 676 and 706 base pairs was found to be the important transcription regulatory site. Four different nuclear were found to bind this region as putative transcription regulators, and remain to be characterized.

SIGNIFICANCE: Major advances in our understanding of key transcription factors which control both pancreatic development and insulin secretion should provide keys to the production of insulin secreting cells which could be transplanted into patients with diabetes. The role of fatty acids in the development of diabetes is becoming increasingly clear. This suggests new therapeutic approaches that should be tested for their efficacy in ameliorating type 2 diabetes. Efforts to understand the inability of the pancreas to compensate for increased insulin resistance could lead to significant breakthroughs in treatment of type 2 diabetes.

FUTURE DIRECTIONS: Studies to explore the role of free fatty acid induced apoptosis in human islets should be a priority. If this mechanism is demonstrated in humans, then studies with inhibitors of nitric oxide production as a means of rescuing the pancreas should be considered. Studies aimed at elucidating the complete pathways in muscle, liver, and fat through which insulin regulates metabolism should be supported. Better methods to assess insulin sensitivity are needed both in humans and in rodent models. Studies aimed at characterizing the differential responsiveness of various tissues to insulin sensitizers as well as the mechanism of action of these drugs should be initiated. In vivo methods to assess β-cell mass would be a tremendous advance in both clinical and basic studies of the pathophysiology of diabetes. The insulin signaling pathway is one of the most heavily studied, but the key elements of this pathway that are involved in diabetes remain elusive. Increased emphasis should be placed on efforts to define the molecules that are primary effectors of insulin action in glucose homeostasis.

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VI. TITLE: Endocrine Regulation of Energy Balance

BACKGROUND: Homeostatic control of body weight and composition is an integrative function of a large number of complex variables resulting in a balance between food intake and energy expenditure. Caloric intake is a result of diet composition, nutrient absorption, cognitive cues, and satiety signals. The partitioning of calories into stored fat, protein, and carbohydrates or conversion to energy through exercise or thermoregulation is intimately controlled by neuronal and endocrine signals similarly to energy intake. Despite this apparent complexity the setpoint for an individual's body weight and lean to fat ratio is remarkably resistant to exogenous influences. When this equation is shifted toward energy storage the resultant obesity is exacerbated by the very same control mechanisms that resist efforts to lower weight. Over the last few years many of the components of this intricate regulatory pathway have been elucidated.

Using positional cloning techniques, NIDDK-supported investigators have made a number of major discoveries in the molecular endocrinology of obesity over the last three years. The identification of the mutations responsible for the obese/diabetic phenotypes of a number of rodent models has revealed a signaling pathway between the hypothalamus and brown and white adipocytes which appears to be a key pathway in the regulation of food intake and energy balance in humans as well as rodents. The discovery of a signaling hormone, leptin, secreted by adipocytes, began an explosion of discovery in the neuroscience and cell biology communities, which has uncovered new elements in the regulation of body composition and energy balance. The discovery of new uncoupling proteins, UCP-2 and UCP-3, in both rodents and humans suggests that thermoregulation may be an important component of the human energy balance equation. The cloning of the leptin receptor and its localization in the hypothalamus and the discovery that melanocortins play a key role in inhibition of food intake have demonstrated the importance of the hypothalamus in this regulatory axis. That a new class of drugs used to treat diabetes, the thiazolidinediones, can work through the nuclear hormone receptor, PPAR-γ, which is also involved in differentiation and function of adipocytes, demonstrates that intervention at any step in this axis can lead to alterations in the delicate balance between food intake, energy storage and engergy utilization. Knock-

outs of melanocortin receptor (MC-4) and β3-Adrenergic receptors have confirmed roles for their cognate ligands in regulation of energy balance. Unexpectedly the knock-out of one of the regulatory subunit isoforms of protein kinase A resulted in a superlean phenotype suggesting that this isoform, which has limited distribution in brain and adipocytes, is involved in intracellular signaling related to energy balance.

The discovery recently of a novel hormone, urocortin, and the localization of this hormone to discrete regions of the brain has led to a number of studies to define its role in control of behavior. Because this hormone is related to CRF (a hypothalamic hormone involved in response to stress) which has potent anorexigenic effects, and urocortin has a much more discrete localization in the brain, Dr, Vale's group investigated the role of urocortin in appetite control. Central administration of urocortin in the brain dramatically suppressed appetite without the concomitant anxiety producing effects of CRF. In addition, urocortin was able to suppress food intake stimulated by NPY treatment. This work suggests that urocortin works through the type 2 CRF receptor, a finding which has implications for the well known effects of CRF-like substances on the immune and cardiovascular systems.

While the necessity to deliver this compound to the brain directly probably limits its therapeutic potential in the treatment of obesity, further studies on the regulation of food intake by urocortin may provide the basis for future therapeutic strategies. In work that may well have immediate impact on drug discovery research, Dr. Roger Cone of the Vollum Institute, has extended his studies on the role of melanocortins in the development of obesity in the agouti mouse. Last year, Dr. Cone reported that the agouti protein is an antagonist at the MSH receptor. This family of receptors, called the melanocortin receptors (MCR), signals for a family of hormones involved in skin pigmentation (MSH) and response to stress (ACTH). Since a subtype of this family named the MC-4 receptor is expressed in hypothalamic regions known to be involved in control of food intake, Dr. Cone developed specific peptide agonists and antagonists to test whether the agouti phenotype could be recapitulated by blocking stimulation of the MC-4 receptor. Treatment of mice with the MCR agonist markedly inhibited feeding in 4 distinct models of hyperphagia whereas treatment with the antagonist at times when feeding is stimulated, such as following a fast or at night greatly enhanced the feeding behavior. Similarly, treatment with the antagonist concomitantly with the agonist blocked the actions of the agonist to inhibit feeding. This work demonstrates that the hypothalamic POMC neuronal system tonically inhibits feeding, and suggests a key role for this site in control of bodyweight.

NIDDK-supported researchers are actively investigating many aspects of adipocyte biology. Included among these efforts are attempts to understand the determinants of adipocyte differentiation, signal transduction in adipocytes, hormones produced by adipocytes, and insulin-induced responses including glucose transport. A number of transcription factors, gene regulatory proteins, have been identified that direct the differentiation of pre-adipocytes to adipocytes. One of these transcription factors, peroxisome proliferator associated receptor-γ (PPAR-γ), is a member of the steroid/thyroid receptor superfamliy. Another, CCAAT/enhancer binding protein (C/EBP), is the founding member of the family of transcription factors called bZIP, named to convey the juxtaposition of a DNA-binding basic (b) region to a leucine zipper (ZIP) dimerization interface that characterizes the family of proteins. PPAR-γ and several C/EBP appear to cooperate to bring about a cessation of mitotic growth and the expression of adipocyte-specific genes that results in the cellular phenotype. Among the genes expressed in adipocytes is GLUT4, which encodes a glucose transporter that cycles between the plasma membrane and cytoplasmic vesicles in an insulin-dependent fashion. Ultimately, control over adipocyte differentiation and metabolism should afford an opportunity to control obesity, a pathological state closely associated with type 2 diabetes.

RECENT FINDINGS: Leptin deficiency appears to account for only a small proportion of human obesity. In the vast major of people with obesity leptin levels are elevated suggesting that a degree of leptin resistance may be involved in the development of obesity. The simplest mechanism of resistance, that is defects in the leptin receptor (as in the db/db mouse) have been reported in humans, but once again appear quite rare. Bjorbaek et al have discovered a novel mechanism in rodents that results in resistance to leptin. The leptin receptor is a member of the cytokine family of JAK-STAT receptors, many of which are involved in the immune system. Taking a cue from work in that field, Bjorbaek looked for an intracellular molecule which has been implicated in suppression of signaling through cytokine receptors. This molecule, SOCS-3 (suppressor of cytokine signaling), indeed appears to be involved in leptin signaling. When expressed in cells SOCS-3 blocks leptin signaling. In various animal models of obesity SOCS-3 is upregulated in hypothalamic cells which contain leptin receptors. Finally, this group showed that leptin treatment in normal animals results in upregulation of SOCS-3 in the hypothalamus. In aggregate, these results implicate SOCS-3 in the leptin resistance observed in most animal models of obesity and suggest that the same mechanism may be involved in human obesity. Left unresolved is whether the increase in SOCS-3 in obesity is secondary to elevation of leptin or is somehow involved in the pathogenesis of obesity.

Whether leptin acts to control food intake or simply informs the hypothalamus of

the amount of energy stored as fat is unclear. Two groups have published results which suggest that food intake may be at least partially independent of leptin. In the first report, Nonogaki and coworkers developed a mouse which lacks the serotonin $5HT_{2C}$ receptor. This receptor is the target for a number of drugs such as dexfenfluramine which have been used to treat obesity. In this animal model hyperphagia (overeating) precedes the development of obesity. The response to exogenous leptin in these animals appears to be normal despite the increased food intake. Leptin resistance develops over time as obesity develops in this model. Despite normal plasma free fatty acids and corticosterone, adipose TNF-α levels were significantly elevated. High levels of TNF-α have been implicated in insulin resistance as well as a number of autoimmune diseases. This model is likely to represent a primary defect in food intake. It is likely that subsequent development of obesity represents a failure of compensatory mechanisms to counterbalance this increase energy intake over a prolonged period. A group lead by Roger Cone at the Vollum Institute has come to a similar conclusion by studying a different brain control pathway. This group crossed mice that produce an endogenous antagonist of the melanocortin receptor in the hypothalamus, the agouti mouse, with mice lacking leptin, ob/ob mice. Whereas agouti mice are leptin resistant, the removal of the endogenous leptin gene by this cross restored leptin sensitivity. While leptin treatment has a marked effect in reducing weight in the ob/ob mouse, when the ob/ob mouse is crossed with the agouti mouse leptin no longer has this effect. Thus, the authors conclude that leptin does not work through the melanocortin system and that the leptin resistance in the agouti mouse is secondary to high leptin levels associated with obesity.

Balancing the inhibition of food intake by inputs through the serotonergic and melanocortinergic pathways are brain peptides that increase food intake. The most notably of these is NPY. This neuropeptide has potent orexigenic effects when injected into the hypothalamus. The lack of a major effect of removal of this peptide through gene knock-out approaches suggests that regulation of food intake involves multiple, redundant pathways. Workers at the Joslin Diabetes Center in Boston decided to test this hypothesis by developing a mouse lacking another hypothalamic peptide which increases food intake, melanin concentrating hormone, or MCH. In contrast to the NPY knock-out mouse, animals deficient in MCH exhibit a marked diminution in food intake along with a significant reduction in weight compared with aged matched controls. These animals lose weight despite reduced leptin levels which would be predicted to increase food intake. Even more strikingly, animals lacking MCH respond to exogenous leptin treatment with reductions in body weight and food intake that parallel that seen in normal animals indicating that the leptin response pathway is functional. Because the presence of this pathway appears obligatory in the stimulation of food intake, identification of the MCH receptor will be of particular

importance. Antagonists at this receptor may be able to control food intake and weight gain without compensation by other regulatory systems.

While much of the work in this field has been focused on regulation within the hypothalamus evidence is increasing that leptin may have a role in other tissues which are important in storage and utilization of energy. Rossetti and coworkers have demonstrated convincingly that leptin is produced in muscle as well as fat. Furthermore, they have demonstrated that both hyperglycemia and hyperlipidemia increase muscle synthesis of leptin. The functional significance of increase muscle leptin synthesis in light of the much greater contribution of fat to plasma leptin levels remains to be elucidated. However, the fact that the increased leptin appears in a physiologically relevant context suggests that it may contribute to energy regulation perhaps locally within muscle.

NIDDK-supported researcher and MERIT recipient, Bruce Spiegelman reported the discovery of a nuclear protein from brown adipose tissue that may be significant to adaptive thermogenesis. The protein, termed PGC-1, is a transcriptional coactivator of certain members of the steroid/thyroid receptor superfamily and its expression is cold-inducible in brown fat and skeletal muscle which are key thermogenic tissues. Previous work from Dr. Spiegelman's laboratory had suggested the presence of a factor in brown fat cells that was not present in fibroblasts which facilitated the transactivation of UCP-1, encoding an uncoupling protein present in mitochondria and linked to energy dissipation. In the recent report, Dr. Spiegelman and colleagues report the isolation of PGC-1 (PPAR-γ Coactivator –1) and its interaction with several members of the steroid/thyroid receptor superfamily including PPAR-γ. The researchers also show that PGC-1 serves as a coativator of PPAR-γ in the transcription activation of UCP-1 in transient transfection assays. Finally, Dr. Spiegelman and co-workers also show that ectopic expression of PGC-1 in white adipose cells induces numerous mitochondrial genes and leads to an increase in mitochondria in the white adipose cells. The latter observation suggests that PGC-1 expression can coordinate a transformation from white adipose to brown adipose. While it remains to be seen whether the transformation includes energy dissipation instead of storage, the finding opens the possibility of combating obesity through manipulation of PGC-1 expression in white adipose.

SIGNIFICANCE: Obesity represents a major health risk in the U.S. inasmuch as it is associated with cardiovascular disease, diabetes and certain forms of cancer. Changes in lifestyle and diet have no doubt contributed to the epidemic of obesity seen in this country over the last few decades. The World Health Organization has recently recognized that obesity is a global menace to health, even in countries where starvation has been the primary concern in the past. As

more and more of the signaling pathways which control energy balance are uncovered, clues to the underlying pathology in obesity are emerging. Each of these regulatory sites provides a potential target for pharmaceutical development.

FUTURE DIRECTIONS: The characterization of regulatory proteins that distinguish energy storing adipocytes from energy dissipating adipocyte may afford the opportunity to develop novel therapeutic approaches to obesity. Research aimed at the isolation and characterization of regulatory proteins in adipose should continue to receive support. The development of a comprehensive database of adipose cDNAs should be considered. Studies aimed at the activation of the adaptive thermogenic pathway in white adipose should be supported. Key regulatory centers in the brain are likely to be good candidates for targeted pharmacotherapies in the treatment of obesity. While Leptin has only proven to be effective in a small number of patients suffering from leptin deficiency, drugs targeted at serotonergic pathways in the brain are widely used today. As the pathways and integration sites within the hypothalamus are uncovered more selective drugs are likely to emerge. A major initiative to define receptors in the hypothalamus should provide impetus for drug discovery efforts in academia as well as industry. The molecular/anatomical substrates for cognitive influences on food intake and energy metabolism should be a focus of investigation.

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VII. TITLE: Molecular Mechanisms Underlying Complications of Diabetes

BACKGROUND: Diabetes mellitus is one of the most prevalent chronic diseases in the United States. Based on the National Health Survey (NHIS), there were 7.8 million diagnosed cases of diabetes in the United States in 1993. It is estimated that about 625,000 new cases of diabetes are diagnosed each year, including 595,000 cases of type 2 diabetes and 30,000 cases of type 1 diabetes. The number of people with diagnosed diabetes increased five-fold between 1958 and 1993. In addition, it is estimated that there are probably 5.4 million undiagnosed cases of type 2 diabetes in the U.S. In the United States, diabetes is a major cause of amputations, blindness, cardiovascular disease and end-stage renal disease. Currently, diabetes is the seventh leading cause of death. In a recent study, it was estimated that the cost of medical care for diabetes in 1992 was $91.8 billion.

The Diabetes Control and Complications Trial (DCCT) conclusively established the relationship between hyperglycemia and the complications of diabetes mellitus. Because of limitations in current therapies, it is often difficult to achieve normal glucose levels in patients with diabetes. Thus, an important therapeutic challenge of diabetes is the prevention and treatment of its chronic complications. However, the detailed sequence of events in the

pathophysiology of complications and the cellular, biochemical and molecular mechanisms that cause diabetes complications have not been elucidated. Several biochemical mechanisms by which hyperglycemia may cause cellular damage have been studied. One theory proposes that hyperglycemia causes an elevation in the activity of the aldose reductase enzyme, which, in turn, results in the abnormal accumulation of simple chemical compounds called polyols. These polyols cause a chain of events that lead to functional and structural dysfunction in the tissues where they accumulate. Another well-developed theory proposes that glucose reacts nonenzymatically with proteins to initiate a modification known as nonenzymatic glycosylation. During this process, the glucose molecules become attached to proteins in blood and cells. This attachment leads to the development of advanced glycation end products (AGEs), which have been implicated in the covalent modification of proteins. It is postulated that such modifications alter the structure of matrix proteins and the function of intracellular proteins, which may lead to diabetic vascular disease. In addition, interaction of an AGE with its specific receptor (RAGE) induces oxidative stress by altering free radical and cytokine production. In animal models, pharmacologic inhibition of AGE formation can prevent diabetic microvascular complications such as retinopathy and nephropathy. Clinical trials of an inhibitor of AGE formation, aminoguanidine, are underway in humans. A third theory attributes the adverse effect of hyperglycemia to the activation of protein kinase C (PKC), a member of the family of serine-threonine kinases that regulate many vascular functions.

Extensive epidemiologic and clinical evidence suggests that, in addition to hyperglycemia per se, genetic determinants are involved in the development of diabetic complications. However, very little is actually known about the identity or function of specific genes involved.

RECENT FINDINGS: Strong evidence exists in animals models that inhibiting the formation of AGEs can prevent or delay the development of microvascular diabetic complications. Now researchers have also shown that AGEs appear to be important in the development of macrovascular lesions in a diabetic mouse model. Investigators were able to prevent the development of accelerated atherosclerotic lesions in diabetic, hyperlipidemic mice by administering a soluble form of the receptor for AGEs.

Administering soluble RAGE prevented AGEs from activating cellular RAGE; in addition, blood and tissue AGE levels decreased, presumably because the AGE: soluble RAGE complex accelerated AGE clearance.

In animal models, microvascular diabetic complications can be prevented or delayed by aminoguanidine. Most studies of aminoguanidine have focused on

its ability to decrease AGE formation. Recently, investigators have demonstrated that aminoguanidine can also act directly as an antioxidant. Aminoguanidine was able to inhibit cell death (apoptosis) caused by hydrogen peroxide in cultured rat retinal cells. Aminoguanidine decreased reactive oxygen species and lipid peroxidation in the cells. In vivo, aminoguanidine decreased lipid peroxide levels in the vitreous of diabetic rabbits.

Clinical and epidemiologic observations suggest that hyperglycemia is not the only factor in the development of long-term complications of diabetes. Thus, some patients with good blood sugar control will develop complications, while, conversely, some patients with poor glycemic control appear to be spared. Previous epidemiologic studies have suggested a genetic influence for the development of diabetic nephropathy. Recently, a study of family members of patients who participated in the DCCT confirmed that familial factors (presumably, genetic) affect the development of nephropathy and demonstrated, for the first time, that familial factors appear to influence the severity of diabetic retinopathy. Further evidence for the role of familial factors in the development of retinopathy comes from data derived from the Third National Health and Nutrition Examination Survey (NHANES III). Analysis of this data revealed an increased risk for development of retinopathy in Mexican Americans with type 2 diabetes compared to non-Hispanic Whites.

SIGNIFICANCE: Much of the past work on the role of AGEs in diabetes complications has focused on microvascular complications. Also of great public health concern is macrovascular disease in diabetes. Patients with diabetes experience an excess risk of heart disease. Heart disease in diabetes occurs earlier in life, affects women almost as often as men, and is more often fatal. Although metabolic factors may influence this increased risk, it now appears that some of the same pathogenetic mechanisms (i.e., AGE formation) are responsible for macrovascular, as well as microvascular, disease. Clearly, this finding has important therapeutic implications.

Aminoguanidine, an inhibitor of AGE formation, has been useful in preventing complications in animals and is currently being studied in humans. The demonstration that aminoguanidine also inhibits oxidative stress has important implications in designing additional pharmacologic agents that might be useful in preventing or treating diabetic complications. In addition, the use of the soluble RAGE receptor to prevent accelerated atherosclerosis in mice provides another potential target for therapeutic intervention.

The long-term complications of diabetes remain a major public health problem. Since any drug carries some risk of side effects, it is imperative to be able to identify those patients with the highest likelihood of developing complications, to

allow targeted interventions. In addition, identifying those populations at highest risk may also lead to the discovery of additional factors and specific genes which determine the development of complications.

FUTURE DIRECTIONS: Further studies are needed to expand our understanding of the role of AGEs, as well as other potentially injurious pathways, in the development of diabetic complications. The molecular pathophysiology of altered protein function and gene expression leading to tissue injury is still unclear. In addition, the possible interrelationships between the various pathways have not been systematically explored. Further refinements in our understanding of the basis for complications will lead to new modalities for the prevention and treatment of these devastating long-term consequences of diabetes. Likewise, it is essential to continue to define population groups at highest risk for the development of specific complications and to identify specific genes and gene products involved in the development of diabetic complications.

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VIII. TITLE: Genetic Syndromes Provide Clues to the Etiology of Diabetes

BACKGROUND: Rare inherited forms of diabetes have provided clues to the genetic causes of the more common sporadic forms of the disease. The first gene identified to cause diabetes was the insulin receptor gene, which caused two phenotypic syndromes, leprechaunism and severe insulin resistance. The syndrome called Maturity Onset Diabetes of the Young (MODY) has also been studied to determine the genetic defects causing this dominantly inherited form of diabetes. MODY subjects exhibit a defect in glucose-stimulated insulin secretion from the β-cell of the pancreas resulting in diabetes before 25-years of age. A large kindred with MODY was studied and the genetic defect was shown to be linked to the adenosine deaminase (ADA) gene on chromosome 20. Surprisingly, other families with MODY did not show linkage with markers on chromosome 20. Studies on these other MODY kindreds identified a second MODY locus (MODY2) on chromosome 7 and a third (MODY3) on chromosome 12. The glucokinase gene was shown to be the genetic cause of MODY2 and mutations were identified in individuals with this form of MODY.

This gene is thought to function as a glucose sensor and regulate insulin release in the β-cell of the pancreas. Last year, the genes responsible for both MODY1 and MODY3 were identified. Using positional cloning and identifying all transcription units in the region, the gene responsible for MODY3 was identified as Hepatic Nuclear Factor - 1α (HNF-1α). Mutations in this gene were demonstrated in individuals from 7 different MODY3 pedigrees. The HNF-1α gene codes for a transcription factor that regulates gene transcription in both the pancreas and the liver. This transcription factor functions as a dimer either with another HNF-1α or the closely related HNF-1β molecule. A dominant/negative effect may be seen if the abnormal subunit binds to a normal subunit inactivating

the entire complex. This dominant/negative effect could explain the dominant form of inheritance seen in MODY. With the identification of an HNF as the genetic defect in MODY3, the HFN-4α gene was investigated as the cause of MODY1 since it was present within the genetic locus on chromosome 20. A single mutation was found to be inherited in the large MODY1 kindred, which would predict a protein of 267 amino acids containing only the DNA binding domain. The HNF-4α, another transcription factor found in both the liver and the pancreas, is a member of the steroid/thyroid receptor supergene family. HNF-4α has been shown to regulate the expression of HNF-1α which may be the mechanism by which it causes MODY.

RECENT FINDINGS: This year, two additional genes have been shown to cause MODY, bringing the number of genes known to cause MODY to 5. Previously, NIDDK-supported researcher and MERIT Recipient Joel Habener had reported a patient with pancreatic agenesis who was homozygous for a mutation in the gene for the insulin promoter factor-1 (IPF-1), more commonly called PDX-1. The specific mutation involved the deletion of a single nucleotide pair in the IPF-1 encoding gene within codon 63 that resulted in premature translation termination. Subsequently, the research group showed that the entire pancreatic agenesis pedigree, who have a diagnosis consistent with MODY, were heterozygous for this mutation. The mutant mRNA encodes two proteins due to the use of a cryptic start site; one corresponding to the N-terminal region of the protein and a second that corresponds to the C-terminal region of the protein. Moreover, the C-terminal domain protein acts as a dominant-negative inhibitor of wild-type IPF-1. Thus, the manifestation of type 2 diabetes in the heterozygotes may not simply be a dosage effect but may be a result of the activity of the dominant-negative protein.

A second family was found to have mutations in the transcription factor HNF-1β, a transcription factor that can dimerize with HNF-1α. Four of the five genes known to cause MODY are pancreatic transcription factors. This finding confirms the importance of the HNF regulatory network in pancreatic β-cell function and in the development of diabetes. Clues for the interaction of these transcription factors were found by studying a patient with MODY3 who had a defect in the promoter region of HNF-1α. This mutation disrupted the binding site for the transcription factor HNF-4α, a factor that causes MODY1 when mutated. The mechanism for the development of MODY3 would appear to be that HNF-4α binding is required to induce HNF-1α.

Two groups have begun to address how defects in HNF transcription factors cause MODY. One paper studies the regulation of a variety of genes involved in glucose homeostasis and found that expression of HNF-4α expression is required for expression of these genes including the glucose transporter 2,

aldolase B, glyceraldehyde-3-phosphate dehydrogenase and liver pyruvate kinase. Another strategy for studying the role of HNF-1α is to analyze the HNF-1α knockout mouse. Originally, the mouse was noted to have defects in liver gene expression. With the discovery that defects in HNF-1α causes MODY, this mouse was examined for signs of diabetes. Studies showed that the heterozygous mouse has elevated glucose levels indicative of diabetes. Studies on insulin release demonstrated a defect in the ability of both glucose and arginine to stimulate glucose secretion. This appears be the mechanism for the elevated glucose levels.

A new genetic syndrome has been described which demonstrates defects in insulin secretion. Mutations in the glutamate dehydrogenase gene have been identified in 8 children with a syndrome of hyperinsulinism-hyperammonemia. These mutations eliminate the ability of the enzyme to be inhibited by GTP and thereby increasing enzyme activity forming excessive amounts of α-keto-glutarate. This syndrome demonstrates the importance of these pathways in regulation of both insulin secretion and ureagenesis.

The gene for another rare syndrome of diabetes called Wolfram syndrome has been identified by positional cloning. The features of this syndrome are diabetes insipidus, diabetes mellitus, optic atrophy and deafness. In this syndrome, the diabetes results from the premature death of theβ-cells. The gene located on chromosome 4 is unique. It is expressed in most of the relevant tissues including the pancreas and brain. It contains 10 regions that may be transmembrane segments. Further studies will be needed to determine the function of this gene in maintaining normal β-cell function.

SIGNIFICANCE: Positional cloning has identified new candidate genes for inherited forms of diabetes. These genes may provide insight into the mechanism for the development of the more common type 2 diabetes.

FUTURE DIRECTIONS: The role of the HNF transcription factors in the development of diabetes needs to be identified. In addition, whether these genes play a role in the more common form of type 2 diabetes needs to be investigated.

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GENETIC METABOLIC DISEASES

IX. TITLE: Cystic Fibrosis: Improved Understanding of the Function of CFTR has Lead to New Insights in Pathogenesis and Treatment of CF

BACKGROUND: Improved therapy has transformed CF from a disease characterized by death in early childhood to a chronic illness, with most patients living to adulthood. In recent years a number of new therapies have been shown to be effective in preventing or retarding the lung disease associated with CF. In 1993, the FDA approved the mucus-thinning drug DNase after research showed this drug reduced the frequency of severe episodes of lung infection and slightly improved lung function after 24-weeks of therapy. In 1995, a randomized controlled clinical trial showed that the anti-inflammatory drug ibuprofen reduced the rate of loss of lung function and improved body weight in patients with CF. In 1997, the FDA approved the use of an inhaled antibiotic which helps control lung infections and reduces the need for hospitalization in patients with CF. Clinical trials are underway to evaluate several other drugs that may prove useful in slowing the progression of lung disease in CF. Improved therapy for pancreatic insufficiency and attention to nutrition has also played a major role in the increased longevity and well being of CF patients.

The clinical presentation of CF can be extremely varied. Mutations, which result in no functional CFTR, cause a classical CF presentation with lung disease and pancreatic insufficiency. Other less severe mutations cause milder forms of the disease often with only some of the symptoms. Understanding how these

mutations cause the pathophysiology of CF requires an understanding of all of the cellular roles of CFTR and its functional domains. This year several new functions of CFTR have been discovered which might lead to a better understanding of the pathophysiology of the disease.

RECENT FINDINGS: The understanding of the pathophysiology of cystic fibrosis relies on the ability to understand the pleotropic effects of the CFTR. Several studies have been published over the past year, which help define some of the disparate cellular functions of this protein. Several new functions of the CFTR molecule have been discovered. In addition, the roles of functional domains that comprise the CFTR protein are being elucidated.

The spectrum of clinical presentations that result from mutations in CFTR has been expanded and is being correlated to the functional properties of CFTR mutations.

One in 25 people are asymptomatic carriers of a mutation in CFTR. A new theory has been proposed to explain the high prevalence of CFTR mutations. A group from the University of Cambridge showed that *Salmonella typhi* uses CFTR to enter intestinal epithelial cells. Mice homozygous for CFTR with ΔF508 mutation showed no uptake of *S. typhi* and heterozygous mice showed a significant reduction. This mutation, which predominates in CF, may reduce susceptibility to typhoid fever leading to a heterozygote advantage.

Studies on another member of the ABC transporter family, has shed new light on the function of many of the CFTR mutations including the most common ΔF508 mutation. The crystal structure of the nucleotide binding domains (NBD) of histidine permease from *Salmonella typhimurium* has been determined. The NBD has been shown to adopt an L-shaped structure with one arm interacting with the adjacent NBD and the other interacting with the membrane-spanning domain. The NBD of most of the ATP transporters has been highly conserved. The most common defect causing CF, ΔF508, occurs in the second arm of the NBD of CFTR, which interacts with the membrane-spanning domains. It may be that disruption of this critical interaction contributes to the mis-folding of this mutant protein. This structure would predict that mutations near the ATP-binding pocket are likely to disrupt ATP binding or hydrolysis where as mutations in arm 1 are likely to impair the ability of the NBD to dimerize.

Recent studies are developing a picture of CFTR functioning in the cell as part of a macromolecular complex. These complex interactions occur with other molecules within the cell including syntaxin, purine receptor, and other chloride-channels. On the cell surface, CFTR interacts with other membrane proteins and these interactions may play a role in fine-tuning CFTR and other channel

activity in response to physiological cues. Understanding how CFTR interacts with other membrane proteins has important implications for design of therapies targeted at activating CFTR or activating other proteins modulated by CFTR to overcome defects in cellular function induced by mutations in CFTR. Recent work identified a membrane protein (syntaxin 1A) that regulates CFTR. Syntaxin 1A was shown to physically interact with the N-terminal of the CFTR chloride channels and regulate movement of chloride both in model systems and in epithelial cells that normally express these proteins. Another protein-protein interacting domain has been discovered at the C-terminal of CFTR. This CFTR PDZ region has been shown to bind the Na+-H+ exchanger regulatory factor, a phosphoprotein that is known to regulate ion transport. Other proteins are likely to interact with this region. Understanding the physiologic regulation of CFTR and other chloride channels has important implications for strategies to activate alternate channels to compensate for defective CFTR function in CF.

Mutations in CFTR have been shown to cause a spectrum of manifestations of CF from a debilitating lung disease with pancreatic insufficiency to a mild form of the disease with congenital absence of the vas deferens (CVD) presenting as infertility due to the absence of sperm. A new clinical presentation has been recognized in this spectrum which consists of chronic pancreatitis without sinopulmonary disease. Two groups studied patients with pancreatic insufficiency of unknown causes. One of these groups supported by NIDDK found that 37 percent of these patients had one abnormal CFTR gene and a subset of these patients had mutations in both CFTR alleles. Because of the numerous CFTR mutations it is not clear at this time whether the patients with only a single identified mutation, in fact, have a second unrecognized CFTR mutation or whether heterozygosity for CFTR contributes to susceptibility to pancreatitis.

Multiple types of mutations have been shown to cause CF. Approximately 10 percent of patients have mutations that insert a stop codon into the CFTR gene resulting in premature termination of the protein. Agents such as gentamicin can cause the protein translation machinery to read through the stop codon resulting in the production of a full-length protein. Administration of gentamicin in a CF cell line has resulted in an increase in CFTR protein and restored cAMP-activated chloride transport. This potential therapy could help the CF patients who harbor this type of mutation.

SIGNIFICANCE: Recent studies have greatly expanded our understanding of the biologic importance of CFTR, the CF gene product. A greater understanding of the role CFTR plays within the cell and the function of its protein domains will lead to new methods to impact this disease.

FUTURE DIRECTIONS: With the discovery of a protein interaction domain within CFTR, studies are ongoing to identify other proteins that interact with CFTR. A complete understanding of the cellular role of CFTR is necessary to understand the contribution of different mutations to the pathophysiology of the disease.

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X. TITLE: Gene Therapy: Understanding the Role of the Immune System In Modulating Gene Expression

BACKGROUND: The ultimate goal for the treatment of genetic diseases is to replace the defective gene with a corrected gene that will remain active for the life of the patient. In theory, this is a simple concept but in reality there are many impediments to targeting a gene to the appropriate cell types and achieving long-term gene expression at physiologic levels.

Encouraging results have been obtained using viral vectors to introduce therapeutic genes into cells. Several different viral systems are being investigated, each with distinct advantages and disadvantages. Initial results with retroviral vectors have shown promise for long-term gene expression, however, the efficiency of transduction, the process by which vector DNA integrates into the cell, is low. In addition, cells must be actively dividing in order for retroviral transduction to occur limiting the application of this technology. Adenoviral vectors provide efficient gene expression in both dividing and non-dividing cells. However, expression has been short lived due to immunological destruction of cells expressing the therapeutic gene as well as viral proteins. Adeno-associated viral (AAV) vectors can transduce non-dividing cells and provide long-term expression but conditions, which are conducive to efficient transduction, need further elucidation. In addition to viral systems, investigators are developing non-viral delivery systems using lipoplexes and receptor-mediated conjugates. These systems have been characterized by transient gene expression but may provide a means of targeting expression to particular cell types. The ideal vector system may combine the advantageous features

from several of the current delivery systems. NIDDK-funded investigators are working on many different aspects of this problem.

One of the major impediments to gene therapy has been immunologic responses to both the viral proteins and to the transgenes that are novel to the host. Individuals can develop a cytotoxic T lymphocyte (CTL) response to these foreign proteins that results in the destruction of cells expressing these genes. This response has been documented with adenoviral vectors. In addition, many individuals have already developed humoral immunity to various viral agents that may interfere with initial attempts at gene therapy. Immune response to viral proteins or the transgenes at the initial administration can also inhibit readministration of the gene therapy vector. Immune response to novel transgenes has been shown to interfere with attempts at therapy. These can occur when a novel transgene such as β-galactosidase is used in the vector or when a therapeutic gene is novel to the host as is the case in null mutations. Significant advances in our understanding of the viral immune response have been reported this year.

RECENT FINDINGS: The observation that an adenoviral vector directly injected into muscle elicits a CTL response but an AAV vector does not, has lead to studies on the mechanism of the immune response to adenoviral vectors. The induction of cellular immunity requires that the foreign protein be presented to the T cell on the surface of an antigen-presenting cell (APC). In addition to presenting the antigen, the APC has several costimulatory molecules such as B7 molecules and CD40 that bind to proteins on the surface of the T cell. NIDDK-supported investigator, James Wilson, has proposed that the mechanism for eliciting an immune response is based on the ability of the vector to transduce dendritic cells. The dendritic cell, also known as the antigen-presenting cell, is efficient at presenting antigen on MHC class I molecules thus activating the T cell response. Direct transduction of the dendritic cell allows efficient presentation of the vector expressed proteins as antigens. Adenoviral vectors efficiently transfect dendritic cells while AAV is a very poor transducer of dendritic cells. Cells transferred from adenoviral infected animals to an animal transfected with the same transgene in an AAV vector will mount a CTL response to the transgene. This process is called adoptive transfer and demonstrates that the transgene in the context of an AAV vector is susceptible to CTL recognition. The inability to transduce antigen-presenting cells appears to be the mechanism by which AAV vectors and their transgene cargo evade the immune system.

Humoral immunity has been shown to be a major component in inhibiting the readminstration of both adenoviral and AAV vectors. The initial administration to a naïve animal results in the appearance of neutralizing antibodies that inhibit

future administrations. One strategy for interfering with the immune response is to block the costimulatory molecules required for activation of the immune response. Two molecules have demonstrated the ability to disrupt this process:CTLA4Ig, a soluble molecule which binds to B7 on the APC blocking the binding of CD28 on the T cell; and MRI, an anti-CD40 ligand antibody that blocks the interaction between the T cell CD40 ligand with both the APC and B cell CD40. Several groups have shown that transient immunosuppression with CTLA4Ig during IV adenoviral vector administration prevented antibody formation and permitted vector readministration. The situation in the lung is more complex since, in addition to systemic immunity, the lung is protected by mucosal immunity. Based on these observations, several immunosuppression regimens have been tested for both AAV and adenoviral vectors. IV administration of CTLA4Ig alone was not able to prevent antibody formation when the vector was administered to the lung. However, two papers from investigators at the University of Washington show that the combination of both CTLA4Ig and MR1, allowed for readministration to the lung. For AAV, a combination of both CTLA4Ig and MR1 administered intraperitoneally resulted in the ability to readminster the vector to the lung. For an adenoviral vector, intraperitoneal administration of both CTLA4Ig and MR1 as well as intratracheal coadminstration of an adenoviral vector expressing CTLA4Ig was required to permit second administration. These immunosuppression regimens also increased the duration of transgene expression.

SIGNIFICANCE: Understanding the role of the immune system in modulating gene expression after gene transfer will allow for the development of more effective methods of gene delivery. Development of a protocol for readministration in the lung will aid in the development of gene therapy for cystic fibrosis.

FUTURE DIRECTIONS: Research to identify new serotypes of AAV for which the population has no prior immunity will be important. This, in combination with a method of immunomodulation like those described, would allow for multiple attempts at gene therapy.

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ENDOCRINE REGULATION OF BONE

XI. TITLE: Clinical Trials of Parathyroid Hormone (PTH) Showing Efficacy as an Anabolic Factor in Bone to Treat Osteoporosis

BACKGROUND: Bone is a dynamic organ, comprised of the extracellular matrix that provides the form and structure of the skeleton and the bone cells that sustain it. While the inert matrix represents both the major structural support for the body and a storage point for important minerals, such as calcium and phosphorus, bone cells must remain continuously active to maintain it. Once bone is formed it undergoes continuous cycles of formation and resorption (breakdown) in response to changing dietary, hormonal, and activity levels. Bone turnover determines bone mass and depends on numerous factors, including mechanical stress, physical activity, diet, and the need to maintain proper serum calcium levels. The balance between bone formation and resorption is regulated by numerous hormones, growth factors, and cytokines, which act upon the bone forming cells (osteoblasts), and bone resorbing cells (osteoblasts). Day-to-day, as well as chronic, fluctuations in serum calcium levels and body activity have important implications for bone mass. Early childhood, adolescence, and late adulthood represent key times during which proper regulation of bone turnover is especially crucial. Early growth and development of the skeleton is central to the development of peak bone mass

into adulthood, while the changes in hormonal balances that occur with aging are important for the maintenance of this peak bone mass through later life. Alterations during both of these periods can have profound effects on the development of osteoporotic fractures later in life.

Steroid hormones, such as vitamin D and estrogen, are particularly important through their actions on dietary supply of the key mineral calcium, as well as actions on bone cells to use these minerals to make bone. Hormones, such as Parathyroid Hormone (PTH) and the PTHrelated Protein (PTHrP) are essential to maintaining the proper balance of minerals between bone and blood. Growth factors, such as insulin-like growth factor-I (IGF-I), are also key players in the regulation of bone turnover at key periods in the life cycle. In postmenopausal women, imbalances in hormones result in imbalances in bone turnover, such that there is a high rate of remodeling of bone, but with a net loss of bone mineral, weakening the bones. This high turnover bone remodeling as a result of loss of natural estrogen results in a rapid and sustained loss of bone mineral, weakening the microscopic structure of the bone to the point at which the risk of fracture is significantly increased. Hormone replacement therapy (HRT) has been used to stem and partly reverse this loss of bone mineral, but side effects, including potential increased risk of breast and uterine cancer limit the utilization of HRT. Considerable effort has focused on development of hormone-like substances that can exert the positive effects of the natural hormone on bone without unneeded or potentially deleterious side effects. Often the interplay in cell signaling responses between and among steroid hormones and growth factors, and other hormones, is an important feature in the regulation of bone turnover. Major progress in developing a complete understanding of how these factors work has been limited by an absence of knowledge of the precise molecular mechanism of action in bone of hormones such as vitamin D, estrogen, PTH and PTHrP.

RECENT FINDINGS: Two small-scale clinical trials testing the efficacy of PTH as an anabolic agent for the treatment of osteoporosis demonstrated that PTH can have a beneficial effect on bone mass. In the first trial, in young women treated with agents to induce a menopause as part of the treatment of endometriosis, PTH 1-34 administered once/day for 12 months increased bone mineral density in the spine, while eliminating further loss of bone mineral at other sites, including the hip and the arm. PTH was well tolerated, with good adherence to the administration regimen. In the second trial in women with secondary osteoporosis due to long-term administration of glucocorticoids in the treatment of chronic inflammatory diseases (e.g. rheumatoid arthritis), 12 months of intermittent PTH treatment results in increased bone mineral density in the lumbar spine, the hip, and the arm. When administered together with estrogen, an even greater effect was observed. Moreover, measurement of biochemical

markers of bone turnover indicated that bone formation had been uncoupled from bone resorption, in favor of continued bone formation. In another study post-menopausal women with osteoporosis and no other interventions received a 2-week trial of intermittent PTHrP 1-36. The drug was well tolerated, and while the duration was too short to demonstrate effects on bone mineral density, biochemical markers of bone turnover were also indicative of a shift to net bone formation.

SIGNIFICANCE: The hormonal regulation of bone turnover has been only incompletely understood. Proper hormonal regulation of bone formation early in development, coupled with other factors such as diet and exercise, combine to allow for the appropriate development of peak adult bone mass. Osteoporosis develops when the regulation of bone turnover is disrupted or in any way seriously impaired. Osteoporotic fractures are a major public health problem, causing significant amounts of pain and disability, primarily in women, but also including men. Hip fractures, in particular, often contribute to early death. With the results of these preliminary small trials hope has been raised that intermittent PTH or PTHrP administration may become a viable therapeutic intervention in osteoporotic women. These new developments in the understanding of hormonal mechanisms underlying the regulation of bone formation on the one hand, and bone resorption, on the other, it becomes possible to develop drugs or other PTH or PTH-rP hormone-like agents (analogs) which can be used at appropriate times to help maintain the proper regulatory balances and potentially rebuild bone. In women, the loss of normal hormonal balances that occur can sometimes be partly reversed through hormone replacement therapy, although for many women there are risks associated with hormone replacement therapy, including a slightly increased risk of the development of tumors in breast and uterus.

FUTURE DIRECTIONS: Further studies to investigate the combination of anabolic hormones with antiresorptive agents, such as Estrogen and the Selective Estrogen Receptor Modulators (SERMS), as well as bisphosphonates are needed to determine whether additive effects on bone mineral density may be obtained. Large studies will be needed to establish the safety and efficacy of these potential new treatments.

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XII. TITLE: Factors Which Mediate Cell Fate Determination of Bone Cell Precursors

BACKGROUND: Cell fate determination during development is a key event necessary for the ultimate development of a mature, functioning cell. Cell signaling at key times in development is essential for the proper expression of a sequence of genes that will determine the fate of that cell. For the bone forming cells or osteoblasts, that precursor, or undifferentiated stem cell, is found in the early mesoderm cells. For the bone resorbing cells or osteoclasts, pluripotent hematopoietic cells in the bone marrow represent the precursor cells. Cell signaling by local growth factors through receptors found on the surface of the undifferentiated cells is translated into a series of intracellular signals which reach the nucleus and either turn on or off important genes. When the full effects of these signals on gene expression are felt, the undifferentiated cell is stimulated to undergo differentiation down a pathway ultimately leading to the mature osteoblast or osteoclast. Often several different signals are required before the fully mature cell is obtained. In most instances the last step in the cascade of cell signaling is the action of a transcription factor in the nucleus, capable of altering the expression of a target gene(s).

RECENT FINDINGS: The osteoclast, or bone resorbing cell, is essential for the

process of bone remodeling. How it is regulated is still unknown, but one component of the process is the recruitment of osteoclast precursor cells to the site of bone resorption and the stimulation of the final stages of differentiation to produce a mature, working multi-nucleated osteoclast. One local cytokine, or growth factor, that has been implicated in osteoclast function is Colony-stimulating Factor-1 (CSF-1). CSF-1 exists in two forms, a soluble form, and a cell surface form. New findings now demonstrate that the cell surface form is capable of stimulating the last stages of differentiation of the osteoclast to form the large, multi-nucleated cells responsible for bone resorption. CSF-1 is, in turn, released by osteoblasts which also express the cell surface form, and do so in response to hormones such as Parathyroid Hormone (PTH), a key mineral regulating hormone, and Tumor Necrosis Factor (TNF), a local bone-active cytokine. It now appears that the cell surface form of CSF-1 when in close proximity to osteoclast precursor cells stimulates osteoclastogenesis, perhaps as part of the cascade of hormonal action stemming from PTH-induced bone resorption. When the cell-surface CSF-1 signal is received, a cascade of cell signaling events triggers the final differentiation of the pre-osteoclast to undergo terminal differentiation to form the mature, working osteoclast.

The osteoblast also arises from pluripotent cells, found in the mesenchyme. Several different local growth factors and bone-active cytokines have been implicated in differentiation of these pre-osteoblast cells. Now it appears that one factor, Bone Morphogenetic Protein-2 (BMP-2) signals mesenchymal precursor cell differentiation to begin the path of development toward becoming an osteoblast. Moreover, two transcription factors within these cells, Smad5 and DPC4 have now been found to mediate the BMP-2 signal in the pre-osteoblast, helping to mediate the final stages in osteoblastic differentiation. Smad5 and DPC4 are found in the cytoplasm of the precursor cells, and are phosphorylated in response to BMP-2 signaling. They then form a dimer complex, which is translocated to the nucleus where gene expression is regulated, with formation of a mature osteoblast the result.

SIGNIFICANCE: These studies have identified important steps in the process of cell fate determination of bone cells. The entire signaling cascade is not known for these cells, but it is now becoming clear that the expression of key signaling molecules at appropriate times in development is required for precursor cells to begin expressing the genes that allow for differentiation, ultimately giving rise to mature functioning bone cells. The regulation of the amounts and activities of these cells helps to determine whether bone will be formed or resorbed, and hence imbalances in cell function contribute to the development of osteoporosis.

FUTURE DIRECTIONS: Elucidation of the exact requirements of signals and signaling molecules may allow for the development of agents which can recruit

undifferentiated precursor cells to sites where bone remodeling is needed. It may then be possible to stimulate formation of bone forming cells to rebuild bone where it has been lost due to osteoporosis.

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BASIC RESEARCH RELEVANT TO DIABETES, ENDOCRINOLOGY METABOLISM

XIII. TITLE: Determination of Specificity in the Hormonal Regulation of Gene Expression

BACKGROUND: Two classes of circulating hormones exist: those that are peptide-based and cannot enter a cell, binding to receptors on the cell surface; and those that are lipid (e.g. steroid)-based and can cross into a cell, binding to receptors in the cell cytoplasm or the nucleus. Signal transduction for the steroid, or nuclear receptor, gene family involves receptors present either initially in the cytoplasm and/or the nucleus, but ultimately binding to target genes in the nucleus. These hormones act as regulators of gene transcription, the final manifestation of endocrine physiology. Often classified by their receptors, the nuclear receptor and steroid hormones include adrenal glucocorticoids and mineralocorticoids, sex hormones (androgens, estrogen, progesterone), vitamin D, thyroid hormone, derivatives of vitamin A (retinoids), and receptors for which hormones have not yet been found, the orphan receptors. Although there are differences in the structures of the different classes of these hormones, their receptors are structurally and functionally similar. In general each receptor has a domain which binds hormone, a dimerization domain which binds to itself or to other receptors to form a dimer pair, a DNA binding domain which binds to specific regions of DNA, and one or more transactivation domains which mediate the effects on the target gene(s). The region of DNA, to which the receptor dimer pair binds, the hormone response element (HRE), acts to either activate or suppress the transcription of the target gene. The enhancer region of the gene does not act alone, but rather requires the presence of a large protein/DNA complex at the actual transcription start site.

In the nucleus, a larger complex of proteins is required to regulate the expression of a target gene. Nuclear accessory proteins are those that either constitutes the transcriptional machinery, itself, or act to regulate the activity of the nuclear receptor. While basal transcription results in limited expression of a gene, higher, or regulated, levels of expression are obtained in response to hormonal signals. These nuclear accessory proteins bind to nuclear receptors or other hormonally regulated transcription factors, and act either as coactivators, or corepressors of receptor dependent gene expression. Specificity is often dependent on whether a particular coactivator complex or corepressor complex is present, and at what levels, in a given cell. While originally discovered and characterized for the thyroid hormone receptor, it is now becoming evident that many nuclear accessory proteins can be found that interact with numerous members of the steroid hormone superfamily. Recent

progress has stressed the role of co-activators, such as Steroid Receptor Co-Activator-1 (SRC-1) which acts to couple the thyroid hormone receptor/retinoic acid X receptor (TR/RXR) dimer pair bound to the promoter region of a gene to the general transcriptional protein complex. These proteins form complexes with other essential regulatory proteins, including CBP (CREB-binding protein), and other similar members of a family of nuclear proteins called the p160 family. All of these proteins are characterized by their ability to bind to hormone receptors, the transcriptional apparatus, and express enzyme activity toward DNA binding proteins. This activity allows for the acetylation or deacetylation of histones, the DNA binding proteins that keep the DNA tightly bound. When acetylated, DNA is "open" allowing for gene expression. When deacetylated, DNA is "closed" and gene expression is repressed. Often receptors from different hormonal pathways interact at the level of the nuclear accessory proteins and the gene in a process called cross talk, and several of these newly discovered nuclear accessory proteins have been found to mediate or act as the agents of this cross talk. The role of the ligand (hormone) is often to either recruit or dislodge a particular nuclear accessory protein, to either release repression or stimulate expression of a gene.

RECENT FINDINGS: Originally, it was felt that only receptors of the steroid hormone superfamily acted through nuclear accessory proteins to modulate gene transcription. Recently, it was shown that other hormonally dependent transcription factors also required nuclear accessory proteins. The POU-domain transcription factors have long been known to act in pituitary cells to regulate development of cell types and expression of genes for key hormones. One such POU-domain transcription factors is Pit-1. Pit-1 has many effects; one such is to regulate the expression of the prolactin gene in pituitary cells. It had also been observed that other hormones, including nuclear receptors, had effects on the regulation of this gene. To determine the mechanism of action of Pit-1 on hormonal gene expression, Rosenfeld and colleagues tested the ability of Pit-1 to interact with a number of nuclear accessory proteins, including CBP. Moreover, they found that phosphorylation of CBP by a number of different hormonal signaling pathways could affect CBP behavior and, consequently, its ability to partner with Pit-1. Rosenfeld and coworkers went on to show that Pit-1 appeared to exist in a balanced relationship with N-CoR and CBP, with the N-CoR complex leading to repression of gene expression and the CBP complex leading to activation of gene expression. The balance between the two was determined by which hormonal signaling pathway was dominant at a given time, with (e.g.) growth factors, such as EGF or insulin, or ligands acting through G-protein coupled receptors leading to activation, and other Growth Factors or hormones and/or nuclear receptors leading to repression. The ultimate effect was either acetylation or deacetylation of DNA-bound histones.

Since any cell is subjected to a myriad of sometimes competing hormonal signals at any time, understanding how ligands, or hormones, affect the balances between receptor-nuclear accessory complexes-DNA is an essential component in understanding the specificity of hormone action. Some of the nuclear receptor family members had all of the appearances of being classical receptors, able to bind DNA, and a ligand, but without a known ligand. One such "orphan" receptor is known as CAR. It was known to be constitutively active, always activating gene expression, but with no known ligand. Now, Moore and coworkers, have found that a naturally present metabolite of androgens (male sex steroids), androstane, acts to turn off CAR activity. It does so by altering the relationship of CAR to nuclear accessory proteins. As a constitutively active receptor, CAR is bound to one class of co-activator, SRC-1. When androstane is present CAR activity is shut down. Androstane apparently binds to CAR, changing its shape slightly and causing it to lose its association with SRC-1. Thus androstane acts as a reverse agonist of hormone action. Thus defining another aspect of selectivity and specificity in hormone action.

Finally, the application of the latest methods of transgenic mouse technology to the question of the role of nuclear accessory factors has shown that when SRC-1 is deleted resultant mice appear nearly normal. There are some rather important defects that become apparent as the animals age, and these relate to the maturation of the sex organs, which are reduced in SRC-1 deficient animals, suggesting a form of partial hormone resistantce. Resistance, in this case, appears to be to estrogen, progesterone, and androgens. The implications of this study are that specificity of hormonal response for the nuclear receptor family may be depend on a given nuclear accessory protein(s).

SIGNIFICANCE: Insight into how hormone signaling results in change in gene expression will open major avenues in the understanding of the hormonal basis of diseases, such as breast and prostate cancer, osteoporosis, and diabetes. Delineating how hormones respond to ligands can help in the design of new therapeutic agents to modulate or control their actions.

FUTURE DIRECTIONS: Further research efforts are needed to fully define pathways of hormonal signaling, specificity of interaction with nuclear accessory factors, and ultimately with target genes in the nucleus.

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XIV. TITLE: Cell Surface Receptors and Regulation of Signal Transduction

BACKGROUND: Cell surface receptors bind hormones that cannot cross into the cell. These peptide-based hormones, growth factors, and cytokines require cell surface receptors to transduce their signals into cellular action. Thus, signal transduction requires the generation of second messengers at the surface of the cell that effect the hormonal signal within the cell. For G-protein coupled receptors (GPCR), a major class of cell surface receptor, the receptor is coupled to guanine nucleotide triphosphate (GTP) binding proteins as the first step in signal transduction. G-proteins then interact with other proteins in the cell to either manufacture cyclic adenosine monophosphate (cAMP), or derivatives of membrane phospholipids-in either case the result is further amplification of the hormonal signal. G-proteins are heterotrimeric complexes made up of 3 proteins, an α-subunit and a dimer of β and γ-subunits. Once G-proteins have been stimulated the α-subunit breaks off and can initiate a

signaling cascade in the cell through interaction with effector molecules, such as adenylyl cyclase (to form cAMP). The β-γ subunit may also initiate signaling cascades. These second messenger-initiated cascades of enzyme reactions amplify the hormonal signal throughout the cell. The ultimate effectors are ion channels, components of the cellular cytoskeletal machinery, or protein complexes, which enter the nucleus and regulate gene expression. The results for the cell can be membrane depolarization, secretion of hormones or other stored products, initiation of the synthesis of new protein(s), initiation of programmed cell death, cell movement, or change in the timing of the cell cycle. Unusual or deranged signaling through GPCRs can also lead to inappropriate cell behavior. If that occurs during embryogenesis it could lead to deformities; if occurring during adult state, it could lead to the development of disease or development of tumors.

RECENT FINDINGS: The GPCR is a large molecule, which loops through the cell surface 7 times. The segments outside of the cell bind the hormone, while the inner segments have roles in signal transduction within the cell. Recently, it was found that the 3rd intracellular segment of the M2- and M3-muscarinic receptors, a class of GPCRs active in muscle, has a specific docking site for one of the G-β/γ dimer complexes, thus providing a platform through which other parts of the signaling cascade can be recruited. This specific docking site may then serve to quickly amplify signal transduction through that class of GPCR.

While there are many known GPCRs, for which a hormone, or ligand, is well characterized, there are others for which a ligand is not known. These receptors are referred to as orphan receptors. Now, it has been shown that the orphan receptor EDG-1, a GPCR implicated in the formation of cell-cell junctions, does have a natural ligand. Sphingosine-1-phosphate is a circulating metabolite that appears to be the natural ligand for EDG-1. When overexpressed, EDG-1 causes very tight cell-to-cell coupling, and when not present, cells that ordinarily couple are unable to do so. When the ligand, sphingosine-1-phosphate, is present it stimulates cell adhesion through activation of a signaling cascade through the EDG-1 receptor. Defects in EDG-1 signaling could be involved in the loss of cell-cell contact that occurs during tumor formation.

One of the consequences of cell signaling through GPCRs is the activation of different classes of protein kinases. A class of kinase that initiates cellular proliferation is the mitogen activated protein kinase family or MAP kinases. One such MAP kinase, the extracellular signal-regulated kinase2 (ERK2) has been implicated in proliferation, programmed cell death, and other functions. New research now shows that when ERK2 is phosphorylated as part of a signaling cascade emanating from GPCRs it forms a dimer pair with another copy of itself and is translocated to the nucleus. There it is able to phosphorylate other

proteins, which are then able to regulate gene expression.

Another mechanism for the coupling of external signals through GPCRs is via the recruitment of small, intracellular G-proteins. This class of G-protein is distinct from the heterotrimeric α-β/γ class. For the rhodopsin receptor, the receptor for light in the rods and cones of the eye and one of the first member of the GPCR family to be identified, a class of small G-proteins has been found to signal in a new pathway that involves an enzyme that acts upon lipids in the cell. This enzyme, phospholipase D, in turn generates a message ultimately leading to changes in cellular calcium concentrations. The release of intracellular stores of calcium serves as a major signal for change in cell action. This new pathway thus explains how certain key cells are able to respond to hormones and other signals. Mutations in these small G-proteins have been implicated in developmental defects and cancer. Understanding how they function to couple signals in cells may help to find means for correcting mutations which lead to disease.

SIGNIFICANCE: Signaling through GPCRs is essential to every day function in most cells. The signaling cascades that flow from these receptors are responsible for cellular changes during development, in response to stress, disease, and signals for programmed cell death. Mutations in such receptors have been implicated in disease and many pharmaceutical agents are designed to selectively stimulate or block signal transduction through GPCRs.

FUTURE DIRECTIONS: Research to fully delineate the mechanisms of signal transduction through GPCRs will prove invaluable in discerning how and why cells become deranged in action and behavior, such as in cancer.

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XV. TITLE: Structure and Mechanism of Electron Transport Chain Proteins

BACKGROUND: The electron transport chain is an essential component of every living organism. It is composed of five protein complexes containing a variety of metal redox centers which are fixed in the inner mitochondrial membrane, plus the lipid soluble molecule ubiquinone and the soluble protein cytochrome c. These complexes facilitate transport of reducing equivalents from NADH and succinate, the products of the TCA cycle, to molecular oxygen, resulting finally in the production of water and the energy transducing molecule ATP. They contain iron-sulfur clusters, heme groups, and copper. The first protein complex I is NADH-ubiquinone oxidoreductase which transfers electrons directly from NADH to ubiquinone. Complex II, or succinate dehydrogenase, transfers electrons directly from succinate to ubiquinone. Complex III, cytochrome bc_1, transfers electrons from ubiquinone to cytochrome c, and complex IV (cytochrome c oxidase) uses these electrons to reduce molecular oxygen to water. The product of the redox cascade is the generation of a proton gradient across the inner mitochondrial membrane, and this electrochemical energy is used by the membrane protein F_1F_0-ATPase to drive ATP formation.

The molecular mechanism whereby the proton gradient is formed is still a matter of some speculation. It is clear from a variety of experiments conducted in intact tissues, isolated mitochondria, and submitochondrial particles that the mitochondrial proton gradient is generated by electron transport, and that use of substrates and oxygen is functionally and tightly coupled to proton gradient

formation and ATP production. Each of the protein complexes in the electron transport chain serve to pass protons across the membrane to build up this gradient, and it is very possible that each protein has its own unique mechanism. The complete structures of these complexes will certainly shed light on this problem, but they have been difficult to obtain because the proteins are hydrophobic and therefore require specialized conditions for crystallization. These complexes tend to contain multiple subunits and are too large to be easily studied with NMR.

RECENT FINDINGS: The complete crystal structure of the membrane protein cytochrome bc_1 from beef and chicken heart was solved to 2.8 A. This structure was reported by three groups within the last year (one supported by NIDDK), and represents a large advance both in the technology of crystallization of membrane proteins and in our understanding of electron transport and proton gradient formation. In fact, the new structure yields a mechanism for the "Q-cycle" theory of proton gradient formation. Cytochrome bc_1 serves to pass electrons from ubiquinone, a redox molecule confined to the lipid interior of the membrane, to cytochrome c, a protein that moves freely on the exterior face of the inner mitochondrial membrane. The redox energy is used to move protons from the matrix side to the cytosolic side of the membrane, forming the proton gradient that is subsequently used to fuel ATP synthesis. The complete complex consists of a dimer of eleven subunits each, with a combined mass of 240 kD.

Cytochrome bc_1 contains three cytochromes (cyt) and one iron-sulphur protein (ISP) which serve as redox centers, in addition to two ubiquinone binding sites which were identified by co-crystallizing the protein with ubiquinone analogues antimycin, myxothiazol and stigmatellin. Cyt b_H and b_L are found within the membrane spanning α-helix region, one near each membrane face. A ubiquinol binding site is associated with each cyt b. The extramitochondrial domain of the protein complex includes cyt c_1 and the nearby cytochrome c binding site, as well as a moveable arm (ISP) with the Fe-S cluster on the free end. This arm crystalized in three different positions dependent on the site of the bound ubiquinone analogue, showing that it can swing between the cyt b_L and cyt c_1 of the opposite monomer, supposedly carrying an electron from one to the other.

These domains support the following "Q cycle" mechanism for coupling proton gradient generation to electron transport. Ubiquinol can accept one or two electrons, adding a proton with each. The fully reduced ubihydroquinone (electrons accepted from NADH or succinate via complexes I and II, and protons pulled from the mitochondrial matrix space) diffuses through the membrane lipid and binds in a hydrophobic pocket near cyt b_L (in the myxothiazol or stigmatellin site near the cytosolic face). Here it forms a hydrogen bond with the moveable Fe-S center. It donates one electron to cyt b_L and one to Fe-S. At the same

time, it gives up two protons to the cytoplasm. Cyt b_L passes its electron down to cyt b_H near the matrix side of the membrane, while the moving arm (no longer attracted to the oxidized ubiquinone) carries its electron up out of the membrane to cyt c_1, which passes it directly to the freely moving cyt c. In the meantime, ubiquinone encounters and accepts an electron from reduced cyt b_H, picking up a proton from the matrix side of the membrane in the process. This mechanism thereby serves to move protons from the mitochondrial matrix to the cytosol using energy released from electron transport down the cytochrome chain.

Structural changes also occur in Complex I (NADH-ubiquinone oxidoreductase) associated with changing redox state. This enzyme contains 48 subunits, with 1 FMN and 5-8 Fe-S clusters. This complex has not yet been crystallized for x-ray diffraction studies. It contains three dissociable domains. The primary NAD(P)H dehydrogenase activity is found in a flavoprotein (FP) domain. The structure was interrogated by subunit cross-linking, or trypsin treatment following binding of the ligands NADPH, NADP or NAD(H). NAD(P) did not alter the pattern of subunit cross-linking or tryptic digestion, but NAD(P)H resulted in a new cross-linking patterns and digestion peptide product. This indicates that conformational changes occur upon reduction of the complex I redox centers, and that these changes expose trypsin sites and alter contact between subunits.

Cytochrome c is a globular protein with one cytochrome molecule that roams the intermembrane face of the inner mitochondrial membrane, passing electrons from cytochrome bc_1 (complex III) to cytochrome c oxidase (complex IV). It is important to understand the protein structural differences between the oxidized and the reduced state in order to interrogate function and the mode of interaction with its redox partners. The structure of oxidized horse heart cytochrome c in solution was found from NMR Nuclear Overhouser Effect and pseudocontact shift experiments. This new structure, obtained in a more physiological preparation, is similar to the crystal structure of the oxidized horse heart and yeast enzymes. The structural changes upon oxidation are subtle, confined to an increased proton lability in the residues around the heme axial ligands, a small rearrangement of one helix near the cytochrome binding site, and slight reorientation of one propionate residue.

SIGNIFICANCE: Despite great advances in understanding the structure-function relationships among molecules throughout the cell, these features of the proteins of the electron transport chain are only now being elucidated. These proteins are very large and contain many subunits, and because they reside in membranes, resist crystalization. Although an enormous amount of indirect evidence allows researchers to propose mechanisms linking proton gradient generation to electron transport, it is only through their structures, and the changes that take place due to reduction/oxidation that we will clearly pin down

these mechanisms.

FUTURE DIRECTIONS: The complete crystal or solution structures of cytochromes I, II and IV, and the F_0 subunit of ATPase remain to be solved. Work will continue toward this end.

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XVI. TITLE: Protein Trafficking in Animal Cells: Sorting Receptors and Membrane Fusion

BACKGROUND: Appropriate delivery of proteins to intracellular destinations and the control of protein maturation and overall abundance are crucial aspects of cellular metabolism. Typically, misfolded proteins fail to travel beyond the endoplasmic reticulum (ER). In fact, the most common mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene for a chloride channel, ΔF508, causes misfolding and retention in the ER. Improper protein trafficking can result in hyperinsulinemia and insulin resistance. In addition, protein processing and trafficking malfunctions are at the root of certain neuroendocrine disorders as well as lysosomal storage diseases (e.g. Niemann-Pick C). Finally, the regulation of protein abundance is also significant to health, since overexpression of protooncogenes is a hallmark of cancer.

The secretory pathway compartments are subdivided into two central membrane populations, the endoplasmic reticulum and the trans-golgi network (TGN). The TGN is crucial to the sorting, export and recovery of soluble and membrane associated secretory proteins. In addition to being the site for an array of important biochemical reactions, the TGN plays a critical role in the routing of proteins to lysosomes, and to the regulated and constitutive exocytic pathways. The routing of membrane and protein traffic between different compartments of the secretory pathway involves the coordinated interaction of many components, including small molecules, lipids, soluble proteins, membrane proteins, and cytoskeletal elements. NIDDK-funded investigators are working to identify novel proteins that regulate membrane and protein traffic in the TGN.

Intracellular trafficking is mediated by vesicles which are comprised of lipid bi-layers that enclose an aqueous compartment. The vesicles emerge from and coalesce with larger subcellular structures that are similarly defined by the lipid bi-layers that enclose their compartments. The mechanism underlying vesicle fusion to target compartments is a focus of research in this area. Vesicles travel in the direction of secretion, anterograde, and in the return, retrograde, direction. Retrograde transport was hypothesized as necessary to preserve the biochemical identity of subcellular compartments, lest they be diluted as a consequence of proteins escaping in vesicles. Indeed, macromolecules have been identified that are subject to retrograde transport and these have allowed discrimination and comparison between vesicles trafficking in opposing directions. Proteins involved in directing traffic have been localized to the exterior of the vesicle in agreement with the expectation that recognition of target compartments by vesicles must occur at their interface in the cytoplasm. Several types of proteins with numerous family members have been implicated in the recognition process including Rabs (GTPases), SNAREs (membrane associated proteins that provide for recognition) and regulators of Rabs and SNAREs. NIDDK-funded investigators are engaged in the effort to determine if specific proteins within each family discriminate the discrete target compartment for each

type of vesicle.

Many proteins, especially secreted hormones, are synthesized in precursor forms and are modified after their initial translation. These changes, including additions, subtractions and three-dimensional folding, are often essential to the biological function of the final product. Investigators funded by NIDDK are active in identifying the enzymes that mediate these post-translational modifications. The molecular mechanisms that account for the specificity of these enzymes as well as accessory proteins that regulate the enzymes are areas of intense study. Cells also monitor the abundance of individual proteins and selectively regulate turnover rates via active destruction. The entities responsible for protein turnover are large structures called proteasomes. The molecular nature of proteasomes and the basis of their selectivity are also under investigation.

RECENT FINDINGS: In the past year, NIDDK-supported researcher Suzanne Pfeffer described the discovery of a cytosolic protein that directs a specific class of transport vesicles to their appropriate cellular destination. Using the recently-developed yeast two-hybrid system for isolating interacting proteins, Dr. Pfeffer identified TIP47, a novel protein that specifically binds to the cytoplasmic tail of mannose 6-phosphate receptors (MPRs). The MPRs are transmembrane proteins that have their tails exposed to the cytoplasm while the balance of the receptor is in the lumen of membrane-enclosed vesicles. The MPRs bind lysosomal hydrolases in the Golgi and, through mechanisms that are not clear, direct these hydrolases to vesicles that will fuse with the early lysosome. After delivering their cargo, MPR-enriched vesicles return to the Golgi for another round of transport. Dr. Pfeffer's work suggests that TIP47 plays a crucial role in this specific transportation pathway. In the report, the group demonstrates that TIP47 binding to vesicles is dependent on the presence of MPRs. Furthermore, depletion of TIP47 from cytosols prevents transport in a cell-free system. Finally, the investigators showed that the expression of antisense RNA against TIP47-encoding mRNA reduced MPR transport in living cells. The discovery and characterization of TIP47 should significantly propel effort to unravel the mechanisms whereby vesicles are appropriately routed to their final destination within the cell. In a paper published in Cell, NIDDK investigator, Nancy Dahms, reports the crystal structure of the cation-dependent MPR. The extracytoplasmic domain of the MPR crystallizes as a dimer, and its structure provides a rationale for the observed differences in binding affinity exhibited by the MPR toward various lysosomal enzymes.

Dr. Gary Thomas, an NIDDK-supported investigator, has identifed PACS-1, a member of a novel gene family that encodes PACS (phosphofurin acidic cluster sorting proteins), as essential for the sorting of proteins to the *trans*-Golgi network (TGN). In this study, the intracellular sorting of the endoprotease furin

was utilized a model system with which to identify the factors that direct protein localization within the TGN. Localization of furin to clathrin-coated regions of the TGN is dependent upon a motif found within furin's cytosolic domain consisting of an acidic cluster (AC) of amino acids. Within the AC are a pair of serines that are subject to casein kinase II phosphorylation. Phosphorylation of these serines within the AC is essential for the TGN localization of furin. PACS-1 is a cytosolic connector protein that binds directly to the TGN localization signal on the furin AC, connecting furin to the clathrin–sorting machinery, and resulting in the localization of furin to the TGN. Cell-free assays demonstrate that the furin AC is not required for TGN budding or retention but functions in a PACS-1-mediated retreival step. Furthermore, PACS-1 is required for the correct localization of the cation independent mannose-6-phosphate receptor, a TGN/endosomal membrane protein that, like furin, is sorted via its AC. PACS-binding motifs are found within many membrane protein cytoplasmic domains, suggesting a broad role for PACS family members in protein sorting within the mammalian secretory pathway.

The delivery of vesicle contents to their appropriate destination requires fusion of the vesicle membrane bilayer with the target organelle's membrane bilayer. Thus, understanding the determinants of membrane fusion is crucial to understanding protein trafficking in a cell. NIDDK-supported researcher and Advisory Council member James Rothman and his colleagues described the minimal machinery for required for membrane fusion in a manuscript published this year in the journal Cell. Rothman and colleagues reconstituted purified, recombinant v- and t-SNARE proteins into separate vesicles. It has been known for some time that the v- and t- SNAREs provide for recognition during membrane fusion. However, these proteins are always found in a large multi-protein complex and their individual roles are not entirely clear. In their recent work, these researchers showed that the v- and t- SNARE proteins alone are sufficient to direct docking and fusion of two membrane-enclosed compartments. The investigators propose a model wherein complementary recognition of v- and t- SNAREs generates a structure similar to viral proteins that direct the fusion of viral membranes with cellular membranes. By analogy to the hairpin structure formed by the viral proteins, Rothman and co-workers have christened the v- and t- complex a SNAREpin. In the model, the viral hairpin and cellular SNAREpin assemble into a configuration that places strain on the proteins. The researchers believe that the energy contained in these strained complexes is harvested to drive membrane fusion. The energy is required to overcome the unfavorable disruption of the membranes' integrity that accompanies fusion of the two membranes. Although the fusion rates of membranes with this minimal machinery is slow, the experimental design should allow the dissection of precisely how other proteins in the multi-protein complex increase fusion rates to those observed in intact cells.

Protein trafficking in a cell occurs not only from the center out to the plasma membrane but in the reverse direction as well. One example is the internalization of plasma membrane receptors. The half-life of these cell surface receptors is determined by controlled internalization and subsequent degradation. NIDDK-supported researcher and Presidential Early Career Award recipient Linda Hicke reported her discovery of the mechanism underlying internalization of well-studied plasma membrane receptor in yeast. Dr. Hicke had previously reported the revolutionary discovery that certain cell surface receptors are targeted for destruction by the same machinery used to target cytoplasmic proteins, the ubiquitin system. In the recent work, Dr. Hicke showed that phosphorylation of the cytoplasmic tail of the receptor is a prerequisite for internalization and subsequent degradation. Many cell surface receptors are phosphorylated on their cytoplasmic domains in response to their activation through ligand binding to extracellular domains. This phosphoryl-ation event is known to be associated with down-regulation of the receptors. Dr. Hicke's recent findings establish the requirement for a specific sequence of amino acids in the cytoplasmic domain to elicit ubiquitination and subsequent internalization in response to receptor phosphorylation. The findings not only describe a specific mechanism for receptor turnover but also establish a connection between cellular signaling events and the ubiquitin-dependent turnover of a cell surface receptor.

SIGNIFICANCE: Ensuring that cells exhibit proper responses after hormonal signaling, environmental stimuli, and during steady-state activity requires that proteins are modified, sent to their proper subcellular compartment, and subjected to appropriate turnover rates. The delivery of proteins to intracellular destinations and the control of protein maturation and overall abundance are fundamental processes of all cells. Knowledge of the proteins involved in the targeting and turnover machinery provides the basis for our understanding of how changes in these processes can lead to metabolic diseases, neuroendocrine disorders, and lysosomal storage diseases.

FUTURE DIRECTIONS: The identification and characterization of proteins involved in directing intracellular trafficking is fundamental to all fields of biomedical research. The number of known traffic destinations suggests that many more recognition and regulatory molecules are waiting to be discovered. The development of in vitro assays such as those that mimic fusion with distinct destination compartments will aid in characterizing proteins involved in intracellular sorting. Further characterization of the ubiquitin-dependent proteasome should improve our ability to predict the relative stability of a given protein. The provision of high resolution structures of individual proteins involved in intracellular trafficking, as well as understanding these proteins in the context of a supramolecular structure is an exciting future direction in this field.

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XVII. TITLE: Hypothalamic-Pituitary Axis Regulation

BACKGROUND: Pioneering work by investigators supported by NIDDK led to the explosion of research in the nascent field of neuroendocrinology in the early 1970s. The discovery of factors released from the brain which in turn regulated

the pituitary gland was revolutionary in the development of our understanding of the coordinated regulation of bodily functions. The discovery of a substance, somatostatin, which inhibits release of GH from the pituitary gland has led to development of current therapies for control of acromegaly as well as diagnostic tools for imaging many cancers. The discovery of the releasing factor that controls reproduction, GnRH, not only led to the Nobel prize for NIDDK investigator Roger Guillemin, but has led to therapies for ovarian and prostatic cancers as well as a potential contraceptive. As, one by one, the hypothalamic factors that control the release of pituitary hormones have been discovered new fields of investigation have been born. NIDDK investigators have led and continue to lead the way in the discovery of the controlling factors involved in almost every important function of the body.

In the early 1980's Guillemin, Wylie Vale, and Michael Thorner discovered the hypothalamic factor that stimulates release of GH from the pituitary. While gargantuan efforts of the scale of the initial studies to purify releasing factors-studies which required literally millions of sheep hypothalami had failed to yield the releasing factors for GH and ACTH, serendipity did not fail. The discovery by Thorner and others that acromegaly could be caused by tumors of the pancreas led to the purification from tumor tissue of The GRF. Of the major regulatory axes which involved the pituitary only the hypothalamic pituitary adrenal (HPA) axis eluded investigators. Corticotropin-Releasing-Factor (CRF) was finally discovered by Vale and coworkers in 1981 opening yet another field to investigators all over the world. This peptide hormone appeared to be widely distributed both in the brain and in peripheral tissues of the body and was shown to have an extremely diverse repertoire of functions from regulation of the adrenal to effects on blood pressure, inflammation, and behavior. Following the discovery of the releasing factors secreted by the brain to regulate growth, reproduction, metabolism, and response to stress through the pituitary gland, literally thousands of investigators have focused on the coordinate regulation of these axes. In each case the subsequent discovery of the receptors for these factors has led to development of both therapeutic and investigational tools that have led to important advances in the clinical management of disease. In 1993, the receptor for CRF was finally identified concurrently by several labs including those of Vale and Michael Rosenfeld. A number of investigators have since worked to elucidate the pathways in the brain which impinge on the pituitary through CRF. One puzzling finding has been that several brain regions which are putatively involved in behavior express CRF but not its receptor as well as the converse. The discovery of Urocortin, a novel brain secretagogue that also works through CRF receptors, as well as the discovery of multiple subtypes of CRF receptors, suggests that there are multiple signaling pathways for this family of proteins which are likely to subserve different functions.

RECENT FINDINGS: To address the possibility that distinct functions of the CRF family of proteins are mediated by each member of the CRF receptor family, Vale and coworkers generated mice lacking the CRF type 1 receptor. Mice lacking this receptor appear relatively normal. It has been proposed that the hypothalamic CRF is necessary for development of the pituitary corticotropes, however, this does not appear to be the case. Basal levels of pituitary ACTH are relatively normal in mice lacking CRF-1 receptors and the adrenal, while quite small, does maintain the ability to secrete glucocorticoids if challenged with ACTH although at very diminished levels. When the animals are exposed to stress they do not show any of the signs of anxiety seen in normal animals. In fact, the mice lacking CRF-1 receptors will exhibit many behaviors that mice normally avoid because they are so stressful. The question remains as to which functions other CRF receptors or CRF-like molecules mediate. This work suggests that organogenesis and development of particular cell types can proceed to a certain point without communication from other parts of the regulatory axis. The programmed development of an organ up to that point appears to be mediated by a family of gene regulatory factors.

One factor, called Prophet of Pit-1 (Prop-1) is the site of the mutation leading to the mouse Ames dwarf phenotype. This factor can activate Pit-1, but only in the absence of another factor called RPX. RPX is expressed in the neural epithelium adjacent to the primordial pituitary gland and disappears at about the time that Pit-1 begins to be expressed. The inactivation of Prop-1 leads to a failure of the presumptive PRL/GH/TSH cells to migrate from the central proliferative zone to their sites of terminal differentiation. A collaboration between basic and clinical NIDDK-supported investigators has now discovered that mutations in Prop-1 can also result in abnormal pituitary development in humans leading to multiple hormone deficiencies. Wu et al demonstrated that familial combined pituitary hormone deficiency (that is, inherited CPHD) can be caused by mutations in Prop-1. An additional finding that was unexpected from extrapolation of the mouse data, was that these patients suffer from lack of gonadotropins in addition to deficits in PRL, GH and TSH. Cogan, Phillips and coworkers, in an international collaboration, have now demonstrated that mutations in Prop-1 are a cause of sporadic CPHD, as well. This group went further to study mutations in Prop-1 in families from many sites around the world. Their data suggest that mutations in Prop-1 occurred independently in these families. Further, their work suggests that DNA symmetry in the Prop-1 gene contributes to the frequency with which this gene is mutated. The finding that mutations in this gene in humans can result in both hypothalamic defects and defects in gonadotropin secretion suggests that this protein has as yet unidentified functions in development.

Taking a cue from work in the fruit fly, investigators recently identified a family of

genes (termed period genes, Per) which are expressed in a circadian rhythm in cells of the suprachiasmatic nucleus in the brain, the so-called circadian clock. Reppert and others have now identified a new member of this gene family, Per3, which raised important questions about how biological rhythms are maintained in mammals. Per1 and Per2 gene are exquisitely sensitive to light exposure to the eye. While the endogenous rhythm of expression of these genes in the brain is different in individual cells, the circadian regulation of multiple functions (for example, sleep) appears to be an integrated response that reflects the mean rhythms of all of the cells. Light, sensed through the retina, can then shift this endogenous rhythm somewhat to conform to the rhythm imposed by the earth's rotation. The mechanism by which individual cells communicate to synchronize their individual circadian rhythms is still unknown. Per3 exhibits an even more intriguing pattern of expression. First, it does not appear to be sensitive to light. Second and even more important, Per3 is widely expressed throughout the body. Per3 maintains a strong pattern of circadian oscillation in these tissues which is not regulated by light. The function of this endogenous clock in peripheral tissues is unknown.

SIGNIFICANCE: Work on neuroendocrine control of body function has broad implications for understanding and treatment of disease. The work by Rosenfeld and others confirms the importance of animal models of development in elucidation of the pathophysiology of human disease. This work also demonstrates that disruption of patterns of gene expression through dysfunction of transcription factors can have a cascade of effects on normal development. The perturbation of normal development of the hypothalamic-pituitary-adrenal axis through elimination of the CRF receptor confirms the role of signaling loops in the directed development of target organs. This latter work also suggests that the CRF type 1 receptor is responsible for mediating the behavioral stress response. By specifying the subtype of receptors involved in this subset of CRF actions, this work may lead to better pharmacotherapies for anxiety. Finally, work by the Reppert lab demonstrating that there are intrinsic circadian rhythms in cells throughout the body not just in the clock cells within the brain, reinforces the concept that application of therapeutic drugs should be based on the knowledge of the intrinsic rhythms of the cells or tissue that are targeted. For example, agents which are designed to kill cells which are actively dividing would be most effective and selective when delivered at a time when those cells are dividing and others are quiescent.

FUTURE DIRECTIONS: The discovery of leptin several years ago spawned an explosion of research into the hypothalamic control of energy balance and refocused attention on this important site of integration for cognitive, behavioral, and metabolic signals. Technological advances now make it possible to identify other novel regulatory pathways through sequencing of gene libraries and high

throughput screening for endogenous ligands. Investigations are also needed to define the essential elements that define a tissue both in its terminal differentiation and in commitment of the primordial tissues prior to evidence of differentiation. Both the mechanism of synchronization between cells with endogenous circadian rhythms and the role of these clocks in peripheral tissues remain to be elucidated. In particular, the function of Period genes in peripheral organs should be a priority since it has implications for both normal function and optimal therapy.

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XVIII. TITLE: Apolipoprotein and Isoprostanes in Lipid Oxidation

BACKGROUND: Lipid metabolism is intimately related to atherosclerosis, which is a major health problem in diabetes and obesity. Atherosclerosis is associated with hypercholesterolemia. Atherosclerotic plaque is currently thought to be initiated when the unsaturated fatty acids in circulating low density lipoproteins (LDL) are peroxidized by reactive oxygen species such as hydrogen peroxide and oxygen radicals. These damaged lipid molecules tend to aggregate, and are recognized by the scavenger receptor of macrophages. Macrophages take up these damaged LDL, and become filled with cholesterol, leading to the formation of "foam cells." These lodge in the smooth muscle wall of arteries and initiate plaque formation. In animal models of athero-sclerosis, the plasma contains autoantibodies against epitopes of oxidized LDL found in atherosclerotic lesions, which likely participate in the associated inflammatory response in the vascular wall.

Low total cholesterol, high HDL cholesterol, and use of exogenous antioxidants are all associated with reduced risk for atherosclerosis. Great progress has been made in identifying the mechanisms responsible for this protection. An important animal model for these studies is the apolipoprotein E-deficient mouse. ApoE binds to the low density lipoprotein receptor (LDLR) with high affinity, which facilitates the LDLR-mediated hepatic lipoprotein uptake and disposal of excess cholesterol in bile. ApoE also binds to cell surface proteoglycans, which sequesters the apoE-containing lipoproteins to the surface of cells such as hepatocytes and vascular endothelial cells. These apoE-deficient animals become severely hypercholesterolemic, and quickly develop atherosclerotic lesions.

Overexpression of HDL apolipoproteins can impact on the atherosclerosis found in apoE$^{-/-}$ mice. Even at elevated total cholesterol concentrations, ApoAI reduces atherosclerotic lesions, while ApoAII increases them. At least some of the protection afforded by apoAI can be attributed to its properties as an antioxidant. After oxidation of isolated HDL particles spontaneous reduction occurs, so that both cholesterol esters and phosphotidylcholine hydroperoxides are reduced to their hydroxide. The methionine residues in the protein component appear to act as the reducer. In fact, canine apoAI has two fewer methionines than the human protein, and is far inferior as a reducer of damaged cholesterol ester. Peptide

Met(O) reductase is present in all mammalian tissues, and may serve to regenerate reduced apoAI.

RECENT FINDINGS: Another apolipoprotein, apolipoprotein AIV (apoAIV), also protects against diet-induced or apoE-deficient atherosclerosis when overexpressed in mice, and may be the most important antioxidant apolipoprotein found in vivo. This protection occurs despite elevated total cholesterol and is independent of changes in HDL cholesterol. ApoAIV is a soluble plasma protein which can also associate with chylomicrons or HDL. It appears to have several biological activities. ApoAIV promotes cholesterol efflux from cells, serves as a ligand for HDL binding to hepatocytes, and may act to stabilize HDL structure or as a cofactor involved in enzymatic remodeling of HDL. It activates lecithin:cholesterol acyltransferase, and modulates the activation of lipoprotein lipase by apoCII. It was recently shown that purified apoAIV protein acts as an antioxidant in vitro, and this may be its major anti-atherogenic property in vivo. ApoAIV was added to isolated lymph or LDL in the presence of either the oxidizing agent copper or peritoneal macrophages, and served to decrease and delay lipid damage and production of thiobarbituric acid-reactive substances. The addition of 2.5 µg/ml apoAIV increased the time of copper-induced conjugated diene formation by 2.4-fold. In comparison, apoE at 20 µg/ml only increased $T_{1/2}$ by 1.75 fold. ApoAIV to interferes with oxidation directly, since addition of the protein after 90 minutes of copper-induced oxidation inhibited further damage to LDL. ApoAIV may be a very important endogenous antioxidant, and since it is mainly produced in the intestine, its production is directly correlated with fat intake. ApoAIV is amphipathic, soluble in both aqueous and lipid environments, and may therefore be able to act as an antioxidant in compartments inaccessible to other protective apolipoproteins.

The predominant oxidized lipids found in human atherosclerotic plaque appear to derive from linoleic acid. On the other hand, these lesions contain highly elevated concentrations of F_2-isoprostanes. Isoprostanes are highly bioactive prostaglandin isomers derived from free-radical peroxidation of arachidonic acid. One of the isoprostanes, $IPF_{2\alpha}$-VI, accumulates in the urine and blood of apoE-deficient mice even before atherosclerotic plaques appear, and may turn out to be an excellent surrogate marker for oxidative stress. When ten-week old $apoE^{-/-}$ mice were fed vitamin E, a potent anti-oxidant, urine and plasma $IPF_{2\alpha}$-VI are reduced to control levels. Most striking was the finding that vitamin E reduced $IPF_{2\alpha}$-VI in the aorta by 50 percent ($p=0.0001$ for correlation), and this was accompanied by a reduction of atherosclerotic lesion from 18.0 percent to 6.2 percent of vascular surface area. Total cholesterol remained well above the level in control mice. Therefore, atherosclerosis was prevented in the presence of elevated cholesterol by the antioxidant vitamin E, which also reduced isoprostane production. This is further evidence that lipid oxidation is a

necessary event in atherosclerotic plaque formation.

SIGNIFICANCE: Atherosclerosis is a major factor in the morbidity and mortality associated with diabetes and obesity. Knowledge of the mechanisms that cause plaque formation, and that protect against it in vivo, will allow the development of new pharmaceutical agents that will increase life expectancy and quality in these patients. Large scale trials of new therapies to prevent or treat atherosclerosis are expensive and time-consuming, but it may be possible to decrease the time and expense by the use of good surrogate markers that can substitute for clinical endpoints. F2-Isoprostanes may be very useful markers of the oxidation damage that initiates and accompanies atherosclerosis.

FUTURE DIRECTIONS: Relatively little is known about lipoprotein metabolism, and our appreciation of the chemical properties of different classes of these molecules is still limited. Further research is needed in order to understand the metabolism, function and compartmentation of apolipoproteins. For those that act as cellular antioxidants, it is important to investigate their mechanism. It will also be important to fully assess whether isoprostanes can be used in the setting of a clinical trial as a surrogate marker for oxidative stress.

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Grant or Contract #	Principal Investigator	Institution
R01DK46405	Hui, D.Y.	Univ of Cincinnati
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R01DK44730	Rokach, J.	Florida Inst of Tech
R01DK48831	Morrow, J.	Vanderbilt Univ

Publication Data:

Cohen RD, Castellani LW, Qiao J-H, Van Lenten BJ, Lusis AJ, Reue K, "Reduced Aortic Lesions and Elevated High Density Lipoprotein Levels in Transgenic Mice Overexpressing Mouse Apolipoprotein A-IV," Journal of Clinical Investigation 1997; 99(8):1906-1916.

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HIV/AIDS-RELATED RESEARCH

XIX. TITLE: Endocrine and Metabolic Disturbances in AIDS

BACKGROUND: Wasting is a frequent AIDS-defining condition, which contributes substantially to the morbidity and mortality of AIDS. In addition to the diminished quality of life and functional impairments associated with wasting, weight loss has been shown to correlate strongly with mortality in AIDS. The pathogenesis of AIDS wasting syndrome is multi-factorial, with secondary diseases, such as infections and gastrointestinal disorders, playing important roles in development of wasting. HIV-positive individuals are often able to maintain stable weight for prolonged periods, with weight loss often occurring episodically in association with secondary infections. Anorexia, a frequent concomitant of infection, impairs the ability to compensate for the increased resting energy expenditure characteristic of AIDS, and weight loss ensues. Unfortunately, neither oral or parenteral nutrition or appetite enhancement with dronabinol or megestrol acetate have been successful in restoring lean body mass in individuals with AIDS wasting syndrome. In an effort to understand the metabolic mechanisms by which lean body mass is lost in AIDS and why, once lost, it has proven so difficult to restore, investigators have examined how HIV affects the anabolic hormones that help accrue and maintain muscle.

RECENT FINDINGS: Wasting imposes a substantial burden of increased morbidity and mortality in individuals infected with HIV. It has been suggested that metabolic alterations are involved in the pathogenesis of AIDS wasting and may prevent lean body mass repletion even when energy intake is adequate. A substantial fraction of men with AIDS have reduced levels of testosterone, a hormone essential for maintenance of muscle mass in men. From thirty to fifty percent of men with AIDS have been reported to have testicular dysfunction (hypogonadism) and low testosterone levels. A recent NIDDK-supported study assessed whether replacement doses of testosterone, administered by means of Androderm, a nonscrotal transdermal patch system, augmented lean body mass, body weight, muscle strength, and health-related quality of life in HIV-infected men with low testosterone. Investigators concluded that Androderm treatment of HIV-infected men with low testosterone levels is safe and is associated with a 1.35 kg gain in lean body mass, a significant reduction in fat mass, an increased red cell count and improvement in emotional measures.

Many of the clinically-important features of HIV/AIDS can be attributed to the immune deficiency which develops in infected patients. The destruction of the immune system by the virus results in opportunistic infections as well as risk of

autoimmune disease and malignancy. Kaposi's Sarcoma (KS), a multifocal tumor of vascular endothelium which typically involves skin and mucosal surfaces, was among the first recognized manifestations of the AIDS epidemic. The KS-associated herpes virus (KSHV) has been implicated in the pathogenesis of KS, but until recently the mechanism by which KSHV triggered KS was unclear. NIDDK-supported researchers have presented three lines of evidence indicating that one protein encoded by the KSHV is a G protein coupled receptor which appears to participate in tumor formation. Investigators at Cornell University have found that this receptor can transform cells to a malignant phenotype. Further they found that expression of this receptor is, by itself, sufficient to cause a switch to an angiogenic (blood-forming) phenotype by stimulating secretion of growth factors and by causing release of enzymes responsible for inducing growth of blood vessels from tissue surrounding the tumor.

SIGNIFICANCE: Favorable changes in body composition, lower cost relative to other anabolic agents, and lower frequency of side effects provide strong rationale for further evaluation of Androderm in the treatment of wasting syndrome. This is the first demonstration that a KSHV encoded gene is capable of both inducing transformation in the infected cells, and stimulating the growth of blood vessels from surrounding tissue which would support tumor growth. These complementary findings strongly support the concept that KSHV infection plays a primary role in the development of Kaposi's Sarcoma. That this work was generated in a laboratory whose primary focus is elucidation of basic mechanisms of G-protein-coupled receptor action underscores the need to continue a strong program in basic research as an essential part of disease based initiatives.

FUTURE DIRECTIONS: Current therapy for the treatment of AIDS has resulted in marked reductions in viral titers in this patients. There have however, been numerous reports of metabolic disturbances including the development of metabolic syndrome in these patients. Studies designed to define the prevalence of this complication in AIDS patients and to characterize these abnormalities should be a high priority of the AIDS program in NIDDK. Ongoing studies on anabolic therapies for the treatment of wasting in AIDS have demonstrated that these therapies may actually have an ameliorative effect on lipodystrophies seen in AIDS patients. As the extent of metabolic disturbances in AIDS patients becomes better defined, potential therapeutic approaches to combat these complications should be explored.

ACKNOWLEDGMENTS:

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Grant or Contract #	Principal Investigator	Institution
R01DK43036	Gershengorn, M.C.	Cornell Univ
R01DK54228	Bhasin, S.	Drew Univ, Ca

Publication Data:

Bhasin S, Storer TW, Asbel-Sethi N, Kilbourne A, Hays R, Sinha-Hikim I, Shen R, Arver S, Beall G, “Effects of Testosterone Replacement with a Nongenital Transdermal System, Androderm, in Human Immunodeficiency Virus-Infected Men with Low Testosterone Levels,” Journal of Clinical Endocrinology and Metabolism 1998;83:3155-3162.

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HIGHLIGHTS OF DDEMD MINORITY PROGRAM ACTIVITIES

XX. TITLE: Type 2 Diabetes Mellitus: Prevention and Treatment

BACKGROUND: Type 2 diabetes mellitus is increasing in prevalence in the United States, with about 7 percent of the adult population affected and 600,000 new cases per year. Type 2 diabetes is even more common in the elderly and in minority populations including African-Americans, Hispanic- Americans, Asian and Pacific Islanders, and Native Americans. In these populations, type 2 diabetes may be present in 10 percent too as much as 50 percent of the adult population. Type 2 diabetes is accompanied by long-term complications including blindness, renal failure, amputations, and a two to four-fold increased risk for cardiovascular disease and stroke. The total health care expenditure for diabetes reflects the high cost of treating the attendant complications and is estimated at approximately one hundred billion dollars or 12 percent of the United States health care expenditure.

In response to the epidemic proportions of type 2 diabetes in the United States, its accompanying long-term complications, and the difficulty of treating type 2 diabetes successfully once it develops, the NIDDK launched the Diabetes Prevention Program (DPP) in 1996 with recruitment to end in May of 1999. The DPP is a randomized multicenter clinical trial to prevent or delay the onset of type 2 diabetes in a high-risk population with impaired glucose tolerance, of which 50 percent will be minority individuals. A cohort of 3000 subjects recruited over a three-year period will be randomized to one of three groups: (1) intensified lifestyle, (2) metformin, and (3) placebo. A fourth troglitazone group has been stopped and the subjects recruited to this intervention arm will be followed off drug for the duration of the study. The study duration will be for 3 to 6-years, with a median duration of 4.5-years. The study is scheduled to end in 2002.

RECENT FINDINGS: Important new information has emerged on the prevalence of diabetes and impaired glucose tolerance in U.S. adults based on the analysis of data from the Third National Health and Nutrition Examination Survey, 1988-1994 (NHANES III) and previous NHANESs. The prevalence of diagnosed diabetes, undiagnosed diabetes, and impaired glucose tolerance in U.S. adults were 5.1 percent, 2.7 percent, and 6.9 percent, respectively. The prevalence of diabetes in adults 40-74-years of age increased from 8.9 percent in the period 1976-1980 to 12.3 percent in 1988-1994. Prevalence was similar in men and women in each racial or ethnic group, but non-Hispanic blacks and Mexican-Americans had 1.6 and 1.9 times greater rates of diabetes compared to non-Hispanic whites. A separate study of the prevalence of glucose intolerance among Native Hawaiians over 30-years of age found the prevalence of diabetes

to be 20.4 percent.

SIGNIFICANCE: The World Health Organization has concluded that an increasing prevalence of diabetes is strongly related to lifestyle. Identification of populations at particular risk for diabetes and testing of interventions to prevent the development of diabetes is essential to reduce the prevalence of diabetes. The DPP will indicate whether lifestyle or pharmacologic interventions can prevent of delay the onset of type 2 diabetes in a study group including 50 percent representation of minorities. Successful strategies to prevent diabetes will reduce the human and financial cost of this disease.

FUTURE DIRECTIONS: Studies are underway to elucidate the basis of increased susceptibility to diabetes in minority populations and to develop interventions tailored to specific populations.

ACKNOWLEDGMENTS:

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Grant or Contract #	Principal Investigator	Institution
NA	Harris, M.	NIDDK
R01DK58136	Mau, M.	U of Hawaii
R01DK02012	Arakaki, R.	U of Hawaii

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Harris MI, Flegal KM, Cowie CC, Eberhardt MS, Goldstein DE, Little RR, Wiedmeyer H-M, Byrd-Holt DD "Prevalence of Diabetes, Impaired Fasting Glucose, and Impaired Glucose Tolerance in U.S. Adults," Diabetes Care 1998;21:518-548.

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XXI. TITLE: Complications of Diabetes Mellitus

BACKGROUND: Diabetes mellitus is one of the most prevalent chronic diseases in the United States. Based on the National Health Survey (NHIS), there were 7.8 million diagnosed cases of diabetes in the United States in 1993. It is estimated that about 625,000 new cases of diabetes are diagnosed each

year, including 595,000 cases of type 2 diabetes mellitus and 30,000 cases type 1 diabetes mellitus. The number of people with diagnosed diabetes increased five-fold between 1958 and 1993. In addition, it is estimated that there are probably 5.4 million undiagnosed cases of type 2 diabetes in the United States, based on fasting plasma glucose levels in representative samples of people without diagnosed diabetes. Of recent concern has been a rapid increase in the number of children and adolescents with type 2 diabetes. This increase has been a particular problem in minority populations. Indeed, certain minority groups, especially African-Americans, Hispanic-Americans and Native Americans, appear to have an increased risk of developing type 2 diabetes.

Chronically elevated blood glucose levels damage tissues and lead to a variety of long-term, potentially disabling complications. In the United States, diabetes is a major cause of amputations, blindness, heart attacks and end stage renal disease. Currently, diabetes is the seventh leading cause of death. In a recent study, it is estimated that the cost of medical care for diabetes in 1992 was $91.8 billion.

The Diabetes Control and Complications Trial (DCCT) conclusively established the relationship between hyperglycemia and the complications of diabetes. The DCCT showed that tightly controlling blood glucose levels is an effective measure to slow the onset of microvascular complications (i.e., retinopathy, nephropathy and neuropathy). However, because of limitations in current therapies, it is often difficult to achieve normal glucose levels in patients with diabetes. Thus, an important therapeutic challenge of diabetes is the prevention and treatment of its chronic complications.

The detailed sequence of events in the pathophysiology of complications and the cellular, biochemical and molecular mechanisms that cause diabetes complications have not been elucidated. Several biochemical mechanisms by which hyperglycemia may cause cellular damage have been studied. However, the exact mechanisms by which elevated glucose levels lead to tissue damage remain incompletely understood.

Extensive epidemiologic and clinical evidence suggests that, in addition to hyperglycemia per se, genetic determinants are involved in the development of diabetic complications. However, very little is actually known about the identity or function of specific genes involved.

RECENT FINDINGS: Clinical and epidemiologic observations suggest that hyperglycemia is not the only factor in the development of long-term complications of diabetes. Thus, some patients with good blood glucose control will develop complications, while, conversely, some patients with poor

glycemic control appear to be spared. Previous epidemiologic studies have suggested a genetic influence for the development of diabetic nephropathy.

Recently, a study of family members of patients who participated in the DCCT confirmed that familial factors (presumably genetic) affect the development of nephropathy and demonstrated, for the first time, that familial factors appear to influence the severity of diabetic retinopathy. Further evidence for the role of familial factors in the development of retinopathy comes from data derived from the Third National Health and Nutrition Examination Survey (NHANES III). Analysis of this data revealed an increased risk for development of retinopathy in Mexican Americans with type 2 diabetes compared to non-Hispanic whites.

SIGNIFICANCE: The long-term complications of diabetes remain a major public health problem. Investigations are currently underway in an attempt to find drugs that would inhibit or reverse complications. Since any drug carries some risk of side effects, it is imperative to be able to identify those patients with the highest likelihood of developing complications, to allow targeted interventions. In addition, identifying those populations at highest risk may also lead to the discovery of additional factors and specific genes which determine the development of complications.

FUTURE DIRECTIONS: Further studies are needed to expand our understanding of the molecular events leading to the development of diabetic complications. Further refinement in our understanding of the basis for complications will lead to new modalities for the prevention and treatment of these devastating long-term consequences of diabetes. Likewise, it is essential to continue to define population groups at highest risk for the development of specific complications and to identify specific genes and gene products involved in the development of diabetic complications.

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N01DK62203	Dahms, W.	Case Western Reserve
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Interagency Agreement		NCHS and NIDDK

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The Diabetes Control and Complications Trial Research Group, “Clustering of

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Harris MI, Klein R, Cowie CC, Rowland M, Byrd-Holt DD, “Is the Risk of Diabetic Retinopathy Greater in Non-Hispanic Blacks and Mexican Americans Than in Non-Hispanic Whites with Type 2 Diabetes?” Diabetes Care 1998;21:1230-1235.

XXII. NIDDK Minority Training and Career Development-FY 1998

Name of Program and Description	Division	# of NIDDK Awards	NIDDK Funding Level	ORMH Collab. Funding
Minority Access to Research Careers (MARC) T-34) NIDDK Co-funds with NIGMS. Funds predoctoral faculty fellowships, visiting scientists, conferences for minority investigators and minority health issues, and honors undergraduate training in biomedical research. Summer Internship Program in the NIDDK Division of Intramural Research (students-managed by NIDDK-EEO).	DK-wide	6	$23,236	
Minority Biomedical Research Support Program (MBRS) NIDDK co-funds with NIGMS. Provides expanded opportunities for minorities to participate in biomedical research careers. Supports research projects of interest to the NIDDK at Minority and Equal Opportunity Institutions.	DK-wide	25	$1,985,728	
R-13 (Conference Grant) to the American Physiological Society. FASEB Provides support for underrepresented minority students to attend meetings of the Society, and for 36 minority high school science teachers to have summer research training in laboratories of Society members.	DK-wide	1	$74,315	

Initiatives for Underrepresented Minorities in Biomedical Research NIH-wide program initiatives to support minority undergraduate, graduate students, high school students, and faculty members on NIDDK active research grants through administrative supplements.	DK-wide	120	$4,500,000	
Research Training of Underrepresented Minorities on Institutional Training Grants (T32) Highly qualified Minority Investigators are assigned T-32 slots held in reserve for this purpose. DDEMD=5 DDDN=3 DKUHD=6	DK-wide	14	$175,174 81,437 183,000	$41,917
Pre-doctoral Fellowships (F-31) To provide support to minority students for research training leading to M.D.-Ph.D. in the biomedical sciences. DDEMD=6 DDDN=2 DKUHD=1	DK-wide	9	$132,269	
Cell/Molecular Biology Student/Teacher Learning Center (R-25) Laboratory Research experience for minorities in the District of Columbia (managed by NIDDK-EEO).	DK-wide	1	$334,767	

<u>Small Research Grants (R-03) for Minority Researchers</u> DDEMD=5 DDDN=n/a DKUHD=1 ORMH Collaboration provides additional support for minority researchers.	 DK-wide ORMH	 6	 $367,229 84,750	 $466,933
<u>Minority High School Student Summer Research Training Supplement</u> In conjunction with the National Minority Organ Tissue Transplant Program award to Howard University, NIDDK provides meaningful laboratory research experience to minority high school students to stimulate their interest in careers in biomedical science.	 DK-wide	 1	 $70,138	
Totals		183	$8,012,043	$508,850